Remarks

Applicants and the undersigned would like to thank the Examiner for her efforts in the examination of this application. Reconsideration is respectfully requested.

I. Objection to the Specification

The Examiner has objected to the Specification as not being enabling.

This objection is respectfully traversed. First, the "Shaking Soda Cans" reference is not material to the present invention. Soda contains carbon dioxide in solution. Absent microbial content, there is no respiration occurring. Shaking a soda can merely hastens the process of bringing gaseous carbon dioxide out of solution.

Stotzky (1965) quotes three published papers dating from 1960 at the latest when stating "It is neither necessary nor desirable to rotate the flask to increase CO_2 absorption." Since this publication, it has become a common practice to determine CO_2 respiration rate in solid and liquid samples. For example, the well-known Warburg manometric respirometer (New Brunswick Scientific, NJ) is equipped with an agitating mechanism to constantly shake the Warburg flasks during an experiment (Umbreit, W.W., 1972. "Constant volume manometry - The Warburg," In: *Manometric Biochemical Techniques*, Umbreit, W. W., R. H. Burris, and J. F. Stauffer (eds.), 5th ed., Burgess Publ. Co. Minneapolis, Minnesota, pp. 1-19). Shaking can get rid of the excess CO_2 in a solution during the pre-incubation. The excess CO_2 in a solution can interfere with the determination of true CO_2 respiration rate, and thus is not desirable in respiration rate determination (e.g., one cannot determine a microbial respiration rate in a soda due to its

excess CO_2 in solution). Therefore, in the present invention, excess CO_2 in the solution is eliminated during the pre-incubation phase by shaking.

In contradistinction to the assertion by Stotzky, shaking does not impair true respiration rate determination in solid as well as liquid samples in most cases. Published respiration data in the literature include shaking during the measurements. For example, Finn et al. (Marine Ecology Progress, Series 243:217-234, 2002, a copy of which is enclosed as Attachment A) states on p. 218: " ... original data [on the respiration of Atlantic cod larvae in sea water] were obtained using a Warburg manometer with agitation during experimentation."

Chicoye (US 4,068,005) is also not material to the present application, since Chicoye are describing fermentation, not respiration, the process and scale of which are entirely different from the microrespiration process addressed in the instant application.

With regard to Experiment #4, FIG. 4 demonstrates that (1) a steady-state CO_2 concentration in the fixed head space of the microrespirometer would be reached if the concentration of alkaline solution is < 0.05 M. (2) The steady-state head space CO_2 concentration is mainly determined by the CO_2 respiration rate, rather than the initial CO_2 concentration of the head space or anything else. For example, if the CO_2 respiration (production) rate of a sample is 10 µL/hr and the initial head space CO_2 concentration is 400 ppm, the alkaline solution in the microrespirometer would absorb CO_2 at a rate of approximately 60 µL/hr according to FIG. 4. Since the absorption rate is much greater than the respiration rate, the CO_2 concentration in the head space will keep decreasing, and so will the absorption rate according to FIG. 4, until it is approximately 40 ppm. At around 40 ppm CO_2, the absorption rate (10 µL/hr) equals the respiration rate and the head space

CO_2 concentration becomes steady as long as the alkaline concentration is not exhausted (practically, at a concentration > 0.00001*M* or pH > 9 or when the indicator is a pink color). At steady state, the CO_2 absorption rate is determined, which is the same as the respiration rate at steady state.

On the other hand, if the respiration rate of a sample is greater than the CO_2 absorption rate at the beginning of the pre-incubation, say, 100 vs. 60 µl/hr, then the head space CO_2 concentration will increase, and so will the absorption rate according to FIG. 4. The CO_2 concentration increase will continue until it reaches approximately 670 ppm, when the CO_2 absorption rate equals 100 µL/hr. Then a steady state is reached again at approximately 670 ppm. At this steady state, the absorption rate is determined to know the respiration rate. Note that the exact head space CO_2 concentration in the whole process does not need to be known; rather, a steady state should be reached and the CO_2 absorption rate determined at the steady state.

The Examiner is further asked to note the research paper enclosed as Attachment B, which discloses substantially the same material as the present application, and which was accepted for publication in a peer-reviewed journal, the *Journal of AOAC International* (**83**(2), 2000, pp. 277-281).

II. Rejection of Claims 2, 3, and 13 under 35 USC 112

The Examiner has rejected Claims 2, 3, and 13 under 35 USC 112 as being nonenabled, and further has rejected Claim 13 as being indefinite.

The issue of whether shaking disturbs the natural evolution rate has been discussed above under §I, wherein it is argued that the data are not distorted by the shaking. Therefore, Claims 2, 3, and 13 are believed to be enabled.

With regard to the indefiniteness of Claim 13, this rejection is respectfully traversed. The initial solution has a concentration of, for example, 1 mM, and at the end, a concentration of 0.01 mM. Therefore, the concentration change is substantially 1 mM (1-0.01 ≈1). Claim 13 has been amended to more particularly point out that which Applicants regard as their invention, namely, to add the units to the left-hand side of the equation (μmol/hr), and also to add that the incremental volume, which is given in an exemplary embodiment as 0.1 mL, is generalized to read *V* for that volume increment.

III. Rejection of Claims 1, 4-10, and 12 under 35 USC 102(b) and Claim 11 under 35 USC 103(a)

The Examiner has rejected Claims 1, 4-10, and 12 under 35 USC 102(b) as being anticipated by Stotzky (*Meth. Soil Anal.*, 1965), and further has rejected Claim 11 under 35 USC 103(a) as being unpatentable over Stotzky. The claims were all commonly owned at the time of the invention.

This rejection is respectfully traversed. Claim 1 has been amended to incorporate the recitations of Claim 2, and Claim 2 has been canceled.

The method described by Stotzky is an open system using a continuous air stream, as opposed to the present invention, which is recited as being carried out "in an enclosed space". The Stotzky method depends on a complete scrubbing of CO_2 in the air stream

to determine the respiration rate, and teaches the maintenance of "a constant flow of air". The present invention does not completely scrub CO_2 at all. In fact, it has to maintain a steady-state CO_2 concentration in the head space by a very dilute alkaline solution (< 0.005 M) in the device, and, again, the head space is recited as being "enclosed".

Further, Stotzky does not determine "from a change in the pH indicator a time increment at which an increment of the alkaline solution is substantially consumed by the carbon dioxide", as there is no volume increment of alkaline solution added in the Stotzky method.

Therefore, Stotzky does not teach every element of the invention as recited in Claim 1, which is required of an anticipation rejection. Nor does Stotzky teach or suggest the invention, but rather teaches away from the claimed invention, as pointed out by the Examiner in her rejection of Claim 2.

Further, the Applicants would like to point out that the Stotzky method requires at least 0.5 M alkaline solution to completely scrub the CO_2 off the air stream. This relatively high concentration of alkaline solution limits the sensitivity of the Stotzky method to about 1.1 mg CO_2 (0.1 mL x 0.5 M x 22 = 1.1 mg) according to the formula in p.1564, or about 560 µL CO_2, as the lower limit of detection. The Stotzky method is not sensitive enough for the real-time (incubation less than 1 hr) determination of respiration rate in the range of 1-200 µL/h (a targeted range of the present invention). The method of the present invention uses very dilute alkaline solution (< 0.005 M) to balance the CO_2 respiration (production) from a sample according to FIG. 4. That is, at a very dilute alkaline concentration (< 0.005 M), the CO_2 absorption rate depends on the CO_2 concentration (FIG. 4) and that ensures a steady state to be established during the pre-incubation period

(please see the explanation above in §I). The detection limit of the present method is about 1 μL/h, two orders of magnitude more sensitive than that of the Stotzky method.

Therefore, for at least these reasons, Claim 1, and Claims 3-12 dependent therefrom, are believed patentably define over Stotzky.

IV. Amendments to Specification and Claim 13

The Specification and Claim 13 have been amended to more particularly point out that which Applicants regard as their invention. In particular, Eq. (2) and its counterpart in Claim 13 have been amended to read:

carbon dioxide evolution rate (μmol/h) = $(V \times 10^3 \times M/2)/(t/60)$,

wherein M is the molarity of the alkaline concentration of the solution, V is a volume of the increment of the alkaline solution in milliliters, and t is the time increment in minutes. No new matter has been entered thereby, and the changes are made to correct the units in the denominator, which are clear from the Specification. The V has been added to replace 0.1, which was also indicated in the Specification a being the volume of the increment added.

Conclusions

Applicants respectfully submit that the above amendments place this application in a condition for allowance, and passage to issue is respectfully solicited. Applicants and the undersigned would like to again thank the Examiner for her efforts in the examination of this application and for reconsideration of the claims as amended in light of the

arguments presented. If the further prosecution of the application can be facilitated through telephone interview between the Examiner and the undersigned, the Examiner is requested to telephone the undersigned at the Examiner's convenience.

Respectfully submitted,

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CERTIFICATE OF MAILING

I hereby certify that the foregoing is being deposited with the United States Postal Service as first class mail in an envelope addressed to the Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450, this 2nd day of August, 2005.

Edward Bradley

Attachment A

Vol. 243: 217–234, 2002 | MARINE ECOLOGY PROGRESS SERIES Mar Ecol Prog Ser | Published November 13

Fuel and metabolic scaling during the early life stages of Atlantic cod *Gadus morhua*

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ABSTRACT: The simultaneous effect of temperature (5, 7, 10 and 13°C) and light on the rates of oxygen consumption and ammonia excretion of larval and early juvenile Atlantic cod *Gadus morhua* was examined. Larvae increased their mean dry body mass by 2000 times within 48 d. Instantaneous growth rate exceeded 30 % d^{-1} towards the end of the study period, and proportionality of growth followed a triphasic pattern, during which body water content significantly declined but no inflection could be detected in the metabolic exponents. Data were rigorously tested via Model-I (least squares) and Model-II (geometric mean) regression techniques, and the aerobic metabolic rate was found to scale allometrically with both dry and wet body mass. The metabolic exponent was not affected by increasing temperature, but was significantly decreased by the presence of light (b = 0.88 to 0.89 for light-adapted larvae; b = 0.90 to 0.91 for dark-adapted larvae). The effect of light on small larvae (4 to 7 mm standard length, SL) caused a 30 to 40 % increase in metabolic rate, while no effect was observed in larger juveniles (40 to 60 mm SL). Acute temperature acclimation of Atlantic cod of 4 to 60 mm SL (0.04 to 350 mg dry mass) demonstrated normal thermal sensitivity with Q_{10} values of 2.4 for dark-adapted larvae and 2.6 for light-adapted larvae. Rates of ammonia excretion also scaled allometrically with wet and dry body mass and showed greater variability in dark-adapted compared to light-adapted larvae. Comparison of the molar rates of ammonia excretion and oxygen consumption revealed that Atlantic cod larvae have a high reliance on amino acids as fuel for energy dissipation. With lipids as the assumed co-substrate, amino acids were estimated to account for 70 to 95 % of total substrate oxidation for larvae up to 7 mm SL (first 3 to 4 wk of post-hatch development). Beyond 7 mm SL, the reliance on amino acids as fuel began to decline, but even in juveniles of 40 to 60 mm SL, amino acids still represented the dominant source of fuel. For juveniles of between 10 and 20 mm SL, both the rates of oxygen consumption and ammonia excretion remained unaffected by the presence of food in the gut. For short-term fasted juveniles (35 to 60 mm SL), however, a substantial decline in the rate of ammonia excretion was observed. This indicates that during short-term fasting (8 to 12 h) early juvenile Atlantic cod conserve amino acids, rather than funneling them into the tricarboxylic acid cycle.

KEY WORDS: Scaling · Metabolism · Fuel preference · Free amino acid · Q_{10} · Temperature · Cod larvae

INTRODUCTION

There is a large volume of literature relating the metabolic rate of animals to their body mass. For vertebrates, the majority of studies have examined these relationships for reptiles, birds and mammals (Hemmingsen 1950, 1960, Zeuthen 1953, 1970, Kleiber 1975, Peters 1983, Schmidt-Nielsen 1984, LaBarbera 1989). The generally accepted, though much debated (Heusner 1982, Feldman & McMahon 1983, Wieser 1984, Riisgård 1998, Witting 1998, Darveau et al. 2002) exponent relating metabolic rate to body mass throughout the animal kingdom is 0.75 (Kleiber 1932,

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1975). For the juvenile and adult stages of fishes, however, a general exponent of 0.8 has been proposed (Winberg 1956, Wieser 1995, Clarke & Johnston 1999). Giguère et al. (1988) have proposed that the metabolic rates of larval fishes scale isometrically with increasing body mass. Post & Lee (1996) also argue for isometric or near-isometric scaling of routine metabolic rate, and they suggest a biphasic ontogeny of metabolism in larval teleosts that is correlated with changing surface-to-volume ratios. Bochdansky & Leggett (2001), however, argue that metabolic scaling of fish larvae follows a curvilinear relationship with increasing body size. Both Giguère et al. (1988) and Bochdansky & Leggett (2001) base their general notions upon interspecific comparisons. Indeed this approach forms the basis of theories that seek to explain the cause of quarter-power scaling in all living organisms (West et al. 1997, 1999, 2001, 2002, Banavar et al. 1999, Enquist et al. 1999, Gillooly et al. 2001, 2002). According to Wieser (1984), however, and more recently to Goolish (1995), Post & Lee (1996) and Darveau et al. (2002), it is important to distinguish between, on the one hand, the intraspecific ontogeny and control of metabolism and, on the other, the interspecific relations of metabolic rate, since failure to do so can confuse the underlying biology of the mass exponent of energy metabolism.

Not all investigators have found an isometric relationship of metabolic rate and body size for the larval stages of fishes (Gruber & Wieser 1983, Wieser & Forstner 1986, De Silva et al. 1986, Bishop & Torres 1999). Moreover, earlier studies of Atlantic cod *Gadus morhua* larvae (Laurence 1978, Serigstad 1987a) have reported metabolic exponents not only of less than unity but of similar magnitude to the juvenile and adult stages studied (Sundnes 1957, Saunders 1963, Edwards et al. 1972). These earlier works, with the exception of Edwards et al. (1972) and Serigstad (1987a), used chemical or manometric techniques to estimate the rate of oxygen consumption. The data of Laurence (1978), remodelled by Laurence (1985), continue to be cited as the basis for models of the metabolism and growth of larval cod (Liesing & Franks 1999, 2000, Buckley et al. 2000). The original data of Laurence (1978) were obtained using a Warburg manometer with agitation during experimentation. As noted by Finn (1994), the manometric method used by Laurence (1973) on larval tautog gave estimates of oxygen consumption that were 4 to 5 times higher than for larvae of other species of fish. A similar observation was also indirectly made by Bochdansky & Legget (2001) for Atlantic cod, when they removed Laurence's (1978) data from their analyses. Further, the currently published models for Atlantic cod are based on a Q_{10} temperature coefficient of 1.07 (Liesing & Franks 1999). Such a value is exceptionally low for fish larvae (Rombough 1988) and requires confirmation.

The Atlantic cod is arguably one of the commercial world's most important species of fish (Kurlansky 1998). As a consequence considerable attention has been placed on its metabolic physiology in order to better understand the life history of this species. Previous studies of juvenile and adult stages of Atlantic cod have investigated the influence of hypoxia, swimming performance, temperature, salinity, and food on the metabolic rate (Soofiani & Hawkins 1982, Soofiani & Priede 1985, Schurmann & Steffensen 1992, 1994, 1997, Bushnell et al. 1994, Nelsen et al. 1996, Steffensen et al. 1994, Tang et al. 1994, Claireaux et al. 1995a,b, 2000, Blaikie & Kerr 1996, Dutil et al. 1997, Reidy et al. 2000). Surprisingly, however, many of these extrinsic and intrinsic factors have yet to be examined for the critically important larval stages. Apart from the studies of Laurence (1978) and Serigstad (1987a), most metabolic studies during the early life stages of Atlantic cod have been confined to the yolk-sac or first-feeding stages (Davenport & Lønning 1980, Davenport et al. 1983, Solberg & Tilseth 1984, 1987, Serigstad 1986, 1987b, Fyhn & Serigstad 1987, Vigrestad 1993, Finn et al. 1995a,b). More recently, Hunt von Herbing & Boutelier (1996) and Hunt von Herbing et al. (2001) were able to correlate the metabolic rate of larval Atlantic cod to different activity events, while Hunt von Herbing et al. (1996a–c) have staged the larvae and early juveniles and provided detailed descriptions of the ontogony of cranial morphology in relation to expected respiratory function.

An important influence on the metabolic rate of fish larvae in the euphotic zone is the presence or absence of light. This especially applies to visual-feeding larvae (Skiftesvik 1992, Cerqueira & Brugger 2001, Downing & Litvak 2001, Utne-Palm & Stiansen 2002). Recent studies have described the advantage of natural light for the growth of Arcto-Norwegian and coastal Atlantic cod (van der Meeren & Jørstad 2001), while other studies have examined the effect of light on the metabolic rate of other larval fishes (Finn et al. 1995c,d, Porter 2001). Apart from the yolk-sac stage studies of Solberg & Tilseth (1984), Serigstad (1987a) and Finn et al. (1995a), no information seems to exist on the important influence of light on the metabolic rate of the early life stages of Atlantic cod. Similarly, the substrate used to fuel metabolism has received little attention in the literature. The only studies published for Atlantic cod larvae are for the cleidoic phase of yolk resorption (Fyhn & Serigstad 1987, Finn et al. 1995a). In the latter study, it was argued that Atlantic cod larvae have a high reliance of amino-acid catabolism as the major foundation of their energy dissipation. At present, however, we remain ignorant of the potential fuel of exogenously feeding larvae.

The initial small size and the rapid growth of Atlantic cod larvae (van der Meeren et al. 1994, Otterlei 2000) make them excellent models for studying the metabolic implications of scaling. Hence, this paper examines the influence of temperature and light on fuel preference and metabolic scaling from first-feeding to beyond metamorphosis of Atlantic cod. The data are intended as an aid to the construction of more accurate models for this important species.

MATERIALS AND METHODS

Two main series of Atlantic cod *Gadus morhua* larvae were followed, each originating from batches of naturally fertilised eggs. Eggs were incubated at 7 to 8°C and transferred to rearing systems on the fourth day post hatching. Larvae were reared under ambient photoperiod (April to June: 60° N, 5° E) and temperature in black-plastic mesocosms (5.2 m^3) on the west coast of Norway near Bergen. Larvae were fed according the feeding rations outlined by van der Meeren et al. (1994) by filtering relevant size fractions of natural zooplankton (mainly *Eurytemora affinis* copepods) into the mesocosms for the duration of the experimental investigation. Prior to respirometry, larvae were transferred to the laboratory and placed in clean (0.2 µm filtered) seawater (32 to 33‰) at the relevant experimental temperature. To examine the effect of acute temperature changes on the routine rates of oxygen consumption ($\dot{V}_{O_2}$) and ammonia excretion ($\dot{V}_{NH_3}$), larvae were fasted (8 to 12 h, depending on size) while acclimating to the experimental temperatures of 7, 10 and 13°C with maximum deviations of ±0.02°C (Series-1 experiments). Temperature maintenance in the laboratory was achieved via water baths using an arrangement of antagonistic thermostats and coolers (Heto), and monitored using a Squirrel (type SQ8-4U, Grant Instruments) data logger. During temperature acclimation, larvae were exposed to ambient light in the laboratory using Luma long-life, standard, colour, fluorescent lamps (36 W/L-73, emitting 70% daylight at 3000 K) which were suspended over the acclimation tanks. Irradiance was determined to be ~50 µE s^{-1} m^{-2} at the water surface using a 180° LI-192SA cosine-corrected underwater quantum sensor attached to a Li-Cor LI 1000 light meter (Li-Cor Environmental). All respiratory measurements were conducted on individual larvae or juveniles. Small larvae were transferred by pipette, while large juveniles were caught by hand in the acclimation vessels (to reduce inclusion of acclimation water) and inserted into the closed respirometers (glass Quickfit FR:S vials) of varying sizes (40 to 500 ml calibrated volume) in an ordered series of 2 blanks, 8 experimental, 2 blanks, 8 experimental, and 2 blanks, such that 16 experimental respirometers could be compared to 6 blank respirometers. All respirometers had previously been filled with aerated clean seawater simultaneously acclimated at each experimental temperature. In addition, ~1 to 2 ml of larval acclimation water was injected into the blank respirometers in order to compensate for the small amount of water associated with the larvae. This procedure was used to allow correction for drift in either the pO_2 or $[NH_3]$, which, if found, was assumed to be linear between blanks. The respirometers were then placed on racks in the thermostat-controlled water bath, where they were exposed to light (10 to 25 µE s^{-1} m^{-2} at the respirometer surface). This procedure was repeated for a second set of 16 experimental and 6 blank respirometers, which were subsequently wrapped in thick black plastic (light transmittance measured to 0 µE s^{-1} m^{-2}) and placed together with the first set of respirometers.

To examine the effect ingested food on the $\dot{V}_{O_2}$ and $\dot{V}_{NH_3}$ (Series-2 experiments), larvae and early juveniles were transferred from their rearing mesocosms to clean filtered seawater at 10°C, 32 to 33‰ as described for the Series-1 experiments. After 1 h of temperature acclimation, individual larvae were inspected under a binocular microscope to ensure the guts contained food organisms prior to inclusion in the respirometers. These experiments were compared to larvae that had been fasted, and acclimated for 8 to 12 h, and that had had no food organisms in the gut during the respirometry. The fed and fasted larvae were examined in the same manner as described for the Series-1 experiments, such that on each Day 16 fed larvae in light and 16 fed larvae in darkness were compared to 16 fasted larvae in light and 16 fasted larvae in darkness at 10°C.

To specifically examine the very earliest phases following the resorption of yolk, a third series of experiments (Series 3) was conducted on larvae at the first-feeding stage. To ensure the correct size range for the first-feeding larvae, eggs stripped and artificially fertilised from 14 female Atlantic cod were incubated in clean seawater (34.5‰) at 5°C under a 12:12 h light: dark photoperiod. Yolk resorption occurred on Day 26 post-fertilisation. On this day, 67 measurements were conducted on groups of 4 to 5 pooled larvae from the different females. Larval rates of $\dot{V}_{O_2}$ and $\dot{V}_{NH_3}$ at 5°C exposed to light (10 to 25 µE s^{-1} m^{-2} at the respirometer surface) were determined.

The $\dot{V}_{O_2}$ was measured by polarographic detection of the oxygen tension (Radiometer E5046 electrode in conjunction with a pH M73 blood-gas analyser) and calculated as the difference between blank and experimental respirometers. The incubation times of the largest juveniles were carefully timed so that the pO_2 did not fall below 6.67 kPa (50 mm Hg), the critical

level at which respiration of Atlantic cod larvae becomes dependent upon available oxygen (Serigstad 1987a). $\dot{V}_{O_2}$ is expressed as nmol ind.$^{-1}$ h^{-1} or as a mass-specific rate ($\dot{V}_{O_2w}$) as µmol g^{-1} h^{-1}. The molar $\dot{V}_{O_2}$ can be converted to the power rate of heat dissipation using a general oxycaloric coefficient of 450 kJ mol^{-1} O_2 (Gnaiger & Kemp 1990). Since the larvae were not hindered in any way within the respirometers, their $\dot{V}_{O_2}$ was assumed to represent the routine rate under the given experimental conditions. The polarographic oxygen sensor was calibrated prior to and after each ordered set of blanks and 8 experimental respirometers, and any drift occurring between calibrations was assumed to be linear. In essence this was essentially every hour, and the maximum recorded hourly drift for any of the experiments was 0.13 kPa (~1 mm Hg). The solubility of oxygen in seawater at each experimental temperature was calculated after Green & Carrit (1967) and Forstner & Gnaiger (1983). The $\dot{V}_{NH_3}$ was determined by sampling 1 ml of the respirometer water (4 replicates) and analysing the [NH_3] according to the procedure of Bower & Holm-Hansen (1980). The molar ratio of $\dot{V}_{NH_3}$ to $\dot{V}_{O_2}$ was used to calculate the apparent nitrogen quotient (*NQ*), which, depending on the co-substrate catabolised, gives an estimate of the mass fraction of amino acids used as fuel (Gnaiger 1983). The calculation assumes that metabolism is fully aerobic and dissipative, as found for turbot (Finn et al. 1995c), but does not account for other forms of excretory nitrogen. Further, since all measurements were conducted on growing, but non-exercising, individuals, the apparent *NQ*s are considered to be a minimum estimate of the relative contribution of amino acids as metabolic fuel.

Following the respiratory measurements, each larva was measured for linear proportions, standard length (SL), and myotome height (MH), as shown in Fig. 2, using a binocular microscope at 6 to 50× magnification for the smaller larvae and a Vernier gauge for the SLs of the larger juveniles. Each larva or juvenile was killed (blow to the head), briefly rinsed in distilled water and carefully blotted with soft tissue paper (Kimwipes®) to remove excess water; it was then placed in preweighed cryotubes (Nunc) for estimation of the wet mass (Sartorius top balance accurate to ±0.1 mg). Larvae were then frozen at −80°C, lyophilised and weighed on a Cahn 25 Automatic electrobalance (accurate to ±1 µg) for estimation of the dry mass. The water content was calculated from the difference between the wet and dry masses.

Data analyses were performed on ln-transformed data, where equations are of the allometric form $y = ax^b$, and on untransformed data, where equations are of the form $y = a + bx$. These structural models were examined using both Model-I (least-squares) and Model-II (geometric-mean) regression techniques and statistically tested via analysis of covariance (ANCOVA) according to the recommendations of Sokal & Rohlf (2000). Significant differences between regressions were examined by Gabriel's approximate T' and GT2 minimum significant difference (MSD) methods outlined by Rohlf & Sokal (1995) and Sokal & Rohlf (2000). Significant differences were only acknowledged if both the MSD and the 95% confidence intervals of the Model-I and Model-II regressions did not overlap.

Interpretations regarding allometric scaling of metabolic rates related to body mass were based on the regression coefficient's significant departure from unity, and for the allometric scaling of body proportions when the regression coefficient significantly departed from 3. This latter interpretation is based on the assumption that body mass reflects the larval volume, which, for isometric growth, varies as the cube of any given linear dimension, and the *y*-intercept passes through zero (Schmidt-Nielsen 1984, Packard & Boardman 1988). Instantaneous growth rate, G (% d^{-1}), was calculated according to the recommendations of Ricker (1958) as $G = (e^g - 1) \times 100$; g is the instantaneous growth coefficient calculated as $[\ln(dM_{t_2}) - \ln(dM_{t_1})]/\Delta t$, where dM_{t_2} and dM_{t_1} are the mean dry masses at times t_2 and t_1, respectively.

The influence of temperature was assessed via the van't Hoff equation for Q_{10} (Eckert 1997). Since the scaling of metabolic rate proved to be allometric, Q_{10} values for individuals of any given size were calculated for temperature intervals of 7 to 10°C and 10 to 13°C using the allometric equations given in Table 2. Since temperature was found not to significantly ($p > 0.05$) affect the metabolic coefficients (b and v) of either light- or dark-adapted larvae, mean regression coefficients of $b = 0.8851$ for light-adapted individuals and $b = 0.9124$ for dark-adapted individuals were used for calculating the Q_{10} values.

RESULTS

Following the onset of exogenous feeding, the closely monitored larvae of Series 1, which had hatched 14 d post-fertilisation, showed an accelerating growth rate over the 48 d of study (Fig. 1). The instantaneous growth rate of Series-1 larvae increased from 7% d^{-1} at the lower temperatures of 7 to 8°C in April, to more than 30% d^{-1} when experiencing the higher temperatures of 14 to 17°C in June. The larvae and juveniles of Series 2 showed growth rates of about 22% d^{-1} between 60 and 80 d post-fertilisation when rearing temperatures increased from 11 to 18°C (temperature data not shown in Fig. 1). This temperature increase was experienced by Series-1 larvae between Days 35

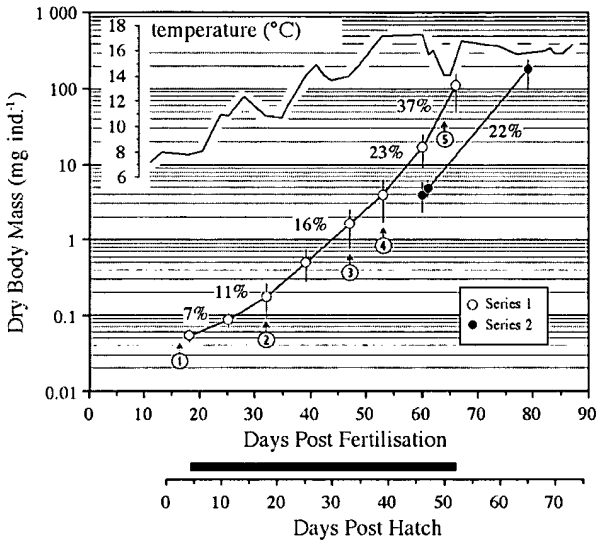

Fig. 1. *Gadus morhua*. Growth (dry mass) of larvae and early juveniles of Atlantic cod reared under natural conditions (April–June; 60° N, 5° E) in a mesocosm and fed natural copepods ad libitum. The recorded temperature for Series-1 experiments is shown above the growth curve. The temperature data for Series 2 is not shown. Data points represent mean ± SD for an N of 42–48 (Series 1) and 44–70 (Series 2). The instantaneous growth rates (% d^{-1}) shown above the growth curve were determined from exponential curves fitted to the data of ranges: 18–25, 25–32, 32–53, 53–60, and 60–66 d post fertilisation, respectively. The circled numbers below the data points represent the 5 larvae depicted in Fig. 2. The shaded bar above the days post-hatch scale represents the 48 d study period of Series-1 experiments

and 50 post-fertilisation when their instantaneous growth rate was 16 to 23% d^{-1}. Both series therefore had similar growth rates under increasing temperature conditions. The more complete data set of Series 1 illustrate that cod larvae, given the right conditions, are capable of increasing their mean dry body mass by 2000 times in just 48 d (0.055 to 110 mg $ind.^{-1}$).

During the course of this rapid growth, the relationship between body mass, MH and SL altered and could be separated into 3 distinct phases (Fig. 2). Following the onset of exogenous feeding and until a SL of 7 mm, larvae grew isometrically ($b \approx 3$) with respect to increasing wet and dry mass as functions of SL (Eqs. 1 & 4; Table 1). Between an SL of 7 to 12 mm, larval growth was allometric ($b \approx 4$, Eqs. 2 & 5; Table 1), and between 12 and 60 mm SL, growth had become near-isometric (b = 3.2 to 3.4, Eqs. 3 & 6; Table 1). These 3 phases of growth were also reflected in the changing MH, although at each phase the relationship between MH and SL remained allometric. During the third phase of growth (12 to 60 mm) the relative body-water content declined significantly ($p < 0.001$, Fig. 3). This was true for both Series-1 and Series-2 juveniles, although the decline in the latter series was greatest.

Despite the tri-phasic pattern of growth and changing body proportions, no inflection could be found in the metabolic scaling of $\dot{V}_{O_2}$ as a function of body mass at any temperature, whether in darkness or exposed to light (Figs. 4 & 5). Furthermore the aerobic metabolic rate scaled allometrically with respect to body mass under all experimental conditions (Eqs. 1–6 & 13–18; Table 2). Rigorous data analysis via the stepwise removal of data points from larger larvae down to 0.1 mg $ind.^{-1}$ dry mass did not yield isometric values for any of the regression coefficients for either Model-I or Model-II regression techniques (b and $v < 1$, $p \ll 0.05$). For larvae between 0.04 and 0.1 mg $ind.^{-1}$ dry mass, the variance increased considerably and regression coefficients were not significantly different from unity. Specific analysis of the $\dot{V}_{O_2}$ of the 14 groups of first-

Table 1. *Gadus morhua*. Biometric scaling constants and statistics for power equations of the form $y = ax^b$, Model-I least-squares regression and $y = ux^v$, Model-II geometric-mean regression. Natural-logarithm-transformed data are modelled for Series-1 larvae. Regression constants are valid for the given size ranges. 95% confidence intervals (CI) and r^2 values are relevant for both Model-I and Model-II regression coefficients (b and v). dM: dry mass; SL: standard length; wM: wet mass; MH: myotome height

Equation	Size range (mm)	N	Model I		Model II		95% CI	r^2
			a	b	u	v		
dM (mg) vs SL (mm)								
(1)	4– 7	115	4.12×10^{-4}	3.109	3.17×10^{-4}	3.262	0.184	0.908
(2)	7–12	80	6.40×10^{-5}	4.129	5.13×10^{-5}	4.229	0.206	0.953
(3)	12–60	127	3.59×10^{-4}	3.406	3.52×10^{-4}	3.413	0.038	0.996
wM (mg) vs SL (mm)								
(4)	4– 7	123	3.84×10^{-3}	2.924	2.50×10^{-3}	3.177	0.224	0.847
(5)	7–12	87	4.60×10^{-4}	4.079	3.76×10^{-4}	4.169	0.187	0.957
(6)	12–60	140	3.59×10^{-3}	3.237	3.54×10^{-3}	3.242	0.029	0.997
MH (mm) vs SL (mm)								
(7)	4– 7	123	3.42×10^{-2}	1.294	2.97×10^{-2}	1.377	0.085	0.883
(8)	7–12	87	6.04×10^{-3}	2.192	5.45×10^{-3}	2.238	0.097	0.959
(9)	12–60	229	3.28×10^{-2}	1.446	3.15×10^{-2}	1.460	0.027	0.981

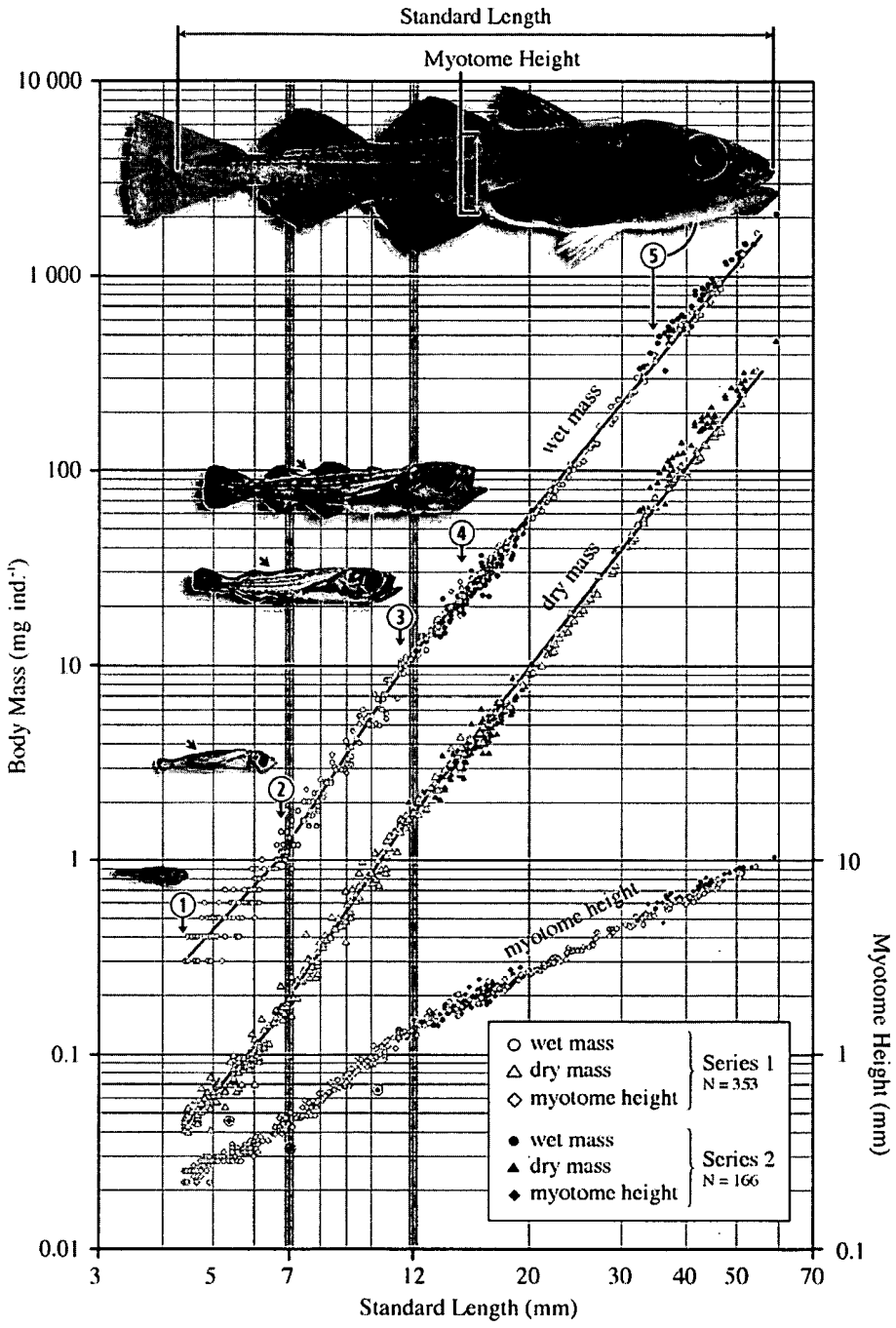

Fig. 2. *Gadus morhua*. Scaling of wet body mass, dry body mass and myotome height in relation to standard length (SL) of larvae and early juvenile Atlantic cod reared under natural conditions (see 'Materials and methods'). Each point represents an individual larva. The natural-logarithm-transformed data of Series 1 are modelled by simple least-squares (Model I) and geometric-mean (Model II) regression of the form $\ln y = \ln a + b \ln x$. These curves are shown fitted in the form of power equations ($y = ax^b$) to the original data of Series 1 on double log-axes. Equations are given in Table 1. Three phases of changing body proportions were found: larvae between 4 and 7 mm grew isometrically ($b \approx 3$), larvae between 7 and 12 mm grew allometrically ($b \approx 4$), while larvae between 7 and 12 mm grew near isometrically ($b = 3.2–3.4$). Photographs of representative larvae are shown to scale, and placed in relation to the x-axis with SLs as follows: ① 4.3, ② 6.8, ③ 11.4, ④ 14.8, ⑤ 35.4 mm. Note that the x-axis is logarithmic. The 3 scaling phases could be recognised by the regression of the fin margin (45° black arrows on larva ②, ③ and ④) and appearance of fin rays

feeding larvae from Series 3 at 5°C (dry mass: 0.04 to 0.07 mg ind.$^{-1}$) also could not demonstrate b values with significant departure from unity (Fig. 6). Over these short size ranges, however, data variance increased considerably, as reflected in the r^2 value (Eq. 33; Table 2) and the 2 regression techniques showed highly divergent results: Model I: $b = 0.990 \pm 0.332$; Model II: $v = 1.648 \pm 0.332$ (Table 2).

The simultaneous effect of light and temperature (Series-1 experiments), revealed discrete metabolic responses with increasing body mass (Fig. 7). The data for $\dot{V}_{O_2}$ of Series-1 larvae exposed to light and darkness revealed that temperature did not significantly affect the slopes of the curves, but caused a parallel upward shift with increasing temperature. Thus, to evaluate the effect of light and temperature, mean regression

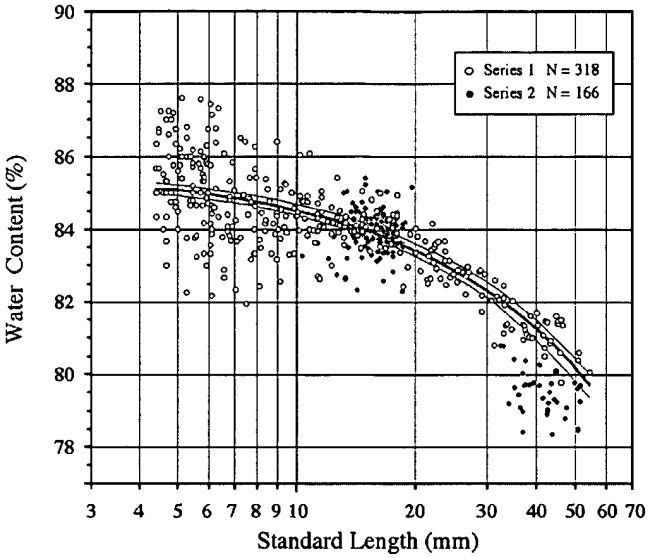

Fig. 3. *Gadus morhua*. Water content (% of wet mass) in relation to SL of larvae and early juvenile Atlantic cod. The curve is fitted (±95% CI) to the untransformed data of Series 1 by Model-I least-squares linear regression

coefficients (b and v) of 0.8851, for larvae exposed to light, and 0.9124, for larvae in darkness were used for further computation. Light was most influential on small larvae, where mass-specific aerobic metabolic rates increased by factors of 1.3 to 1.4 times the rate in darkness at any given temperature. The stimulatory effect of light gradually declined, however, until no significant effect could be detected in the largest juveniles, as reflected in the shallower slope of the $\dot{V}_{O_2}$ of the light-adapted individuals. The Q_{10} estimates of the effect of temperature on routine metabolic rate were calculated for mean metabolic exponents of light- and dark-adapted larvae. The Q_{10} values were found to decrease with increasing temperature such that values for light-exposed larvae ranged from 2.53 to 2.61, with a mean of 2.57, while Q_{10} values for larvae in darkness ranged from 2.36 to 2.46, with a mean of 2.40.

Allometric scaling relationships were also established for the larval rates of ammonia excretion ($\dot{V}_{NH_3}$) versus dry and wet mass in both light and darkness at the 3 experimental temperatures (Figs. 4 & 5). The variablity of the $\dot{V}_{NH_3}$ data were greater than for $\dot{V}_{O_2}$, particularly for the dark-adapted larvae. Regression analysis and ANCOVA revealed that the slopes did not differ ($p > 0.05$) over the full range of dry and wet masses of light-adapted larvae (Eqs. 7–9 and 19–21, Table 2). This also proved true for the dark-adapted larvae, but only for larvae with dry masses falling in the range of 0.08 to 100 mg ind.$^{-1}$ and wet masses between 0.3 and 530 mg ind.$^{-1}$ (Eqs. 10–12 & 22–24; Table 2). Specific analysis of the $\dot{V}_{NH_3}$ of the 14 groups of first-feeding larvae from Series 3 at 5°C (dry mass: 0.04 to 0.07 mg ind.$^{-1}$) showed significant differences between the regression coefficients of Model-I and Model-II methods. The regression coefficient of Model I did not reveal a significant departure from isometric

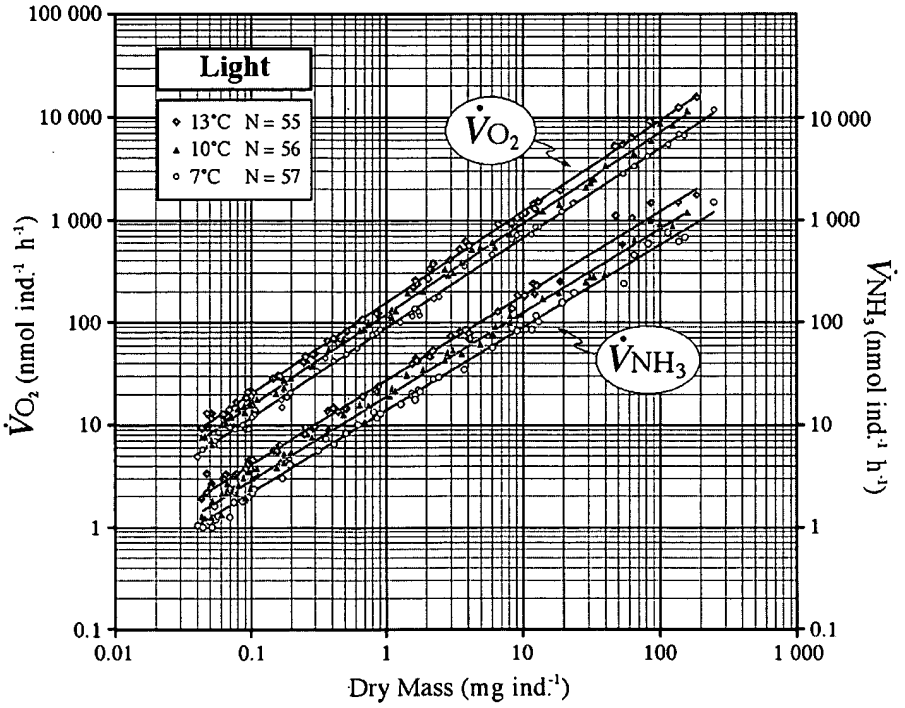

Fig. 4. *Gadus morhua*. Routine rate (nmol ind.$^{-1}$ h^{-1}) of oxygen uptake ($\dot{V}_{O_2}$) and ammonia excretion ($\dot{V}_{NH_3}$) related to dry mass of individual short-term-fasted larvae and early juvenile Atlantic cod (Series 1) at 3 temperatures exposed to light of 10–25 µE s^{-1} m^{-2} at the respirometer surface. The fasting period varied from 8 to 12 h depending on larval size. The curves are modelled as described for Fig. 2 and shown fitted to untransformed data on double-logarithmic axes

Table 2. *Gadus morhua*. Metabolic scaling constants and statistics for power equations of the form $y = ax^b$, Model-I least-squares regression and $y = ux^v$, Model-II geometric-mean regression. Data are analysed as described for Table 1. Eqs. (1)–(24) refer to Series-1 fasted larvae. Eqs. (25)–(32) refer to Series-2 experiments of fed and fasted larvae: F/light, fed larvae exposed to light; F/dark, fed larvae in darkness; S/light, fasted larvae exposed to light; and S/dark, fasted larvae in darkness. Eqs. (33) & (34) refer to Series-3 fasted first-feeding stage larvae exposed to light. The amount of photon energy at the respirometer surface for light-adapted larvae was 10–25 µE s^{-1} m^{-2}

Equation		T (°C)	Size range	N	Model I		Model II		95% CI	r^2
					a	b	u	v		
SERIES 1 EXPERIMENTS										
$\dot{V}_{O_2}$ (nmol ind.$^{-1}$ h^{-1}) vs dry mass (mg ind.$^{-1}$)										
(1)	Light	7	0.04–250	57	88.59	0.878	88.58	0.879	0.010	0.998
(2)		10	0.04–200	56	116.97	0.885	116.96	0.886	0.010	0.998
(3)		13	0.04–200	55	155.94	0.889	155.93	0.890	0.010	0.998
(4)	Dark	7	0.04–225	55	73.50	0.917	73.50	0.918	0.014	0.997
(5)		10	0.04–150	43	96.34	0.904	96.33	0.906	0.020	0.995
(6)		13	0.04–150	52	124.40	0.917	124.37	0.918	0.014	0.997
$\dot{V}_{NH_3}$ (nmol ind.$^{-1}$ h^{-1}) vs dry mass (mg ind.$^{-1}$)										
(7)	Light	7	0.04–250	57	14.17	0.805	14.17	0.855	0.037	0.975
(8)		10	0.04–200	55	19.26	0.817	19.26	0.873	0.035	0.983
(9)		13	0.04–200	54	27.64	0.823	27.63	0.859	0.024	0.990
(10)	Dark	7	0.08–100	41	12.31	0.818	12.28	0.824	0.031	0.986
(11)		10	0.08–100	40	16.57	0.844	16.55	0.852	0.040	0.981
(12)		13	0.08–100	43	21.88	0.853	21.85	0.857	0.029	0.990
$\dot{V}_{O_2}$ (nmol ind.$^{-1}$ h^{-1}) vs wet mass (mg ind.$^{-1}$)										
(13)	Light	7	0.3–1300	61	16.75	0.908	16.71	0.909	0.012	0.998
(14)		10	0.3–820	61	21.92	0.909	21.86	0.910	0.012	0.997
(15)		13	0.3–920	61	28.67	0.915	28.60	0.916	0.013	0.997
(16)	Dark	7	0.3–1140	57	13.18	0.938	13.12	0.940	0.017	0.996
(17)		10	0.3–660	55	18.49	0.918	18.39	0.921	0.021	0.993
(18)		13	0.3–800	57	22.16	0.939	22.05	0.942	0.019	0.995
$\dot{V}_{NH_3}$ (nmol ind.$^{-1}$ h^{-1}) vs wet mass (mg ind.$^{-1}$)										
(19)	Light	7	0.3–1300	61	3.07	0.834	3.05	0.837	0.018	0.997
(20)		10	0.3–820	61	4.07	0.841	4.05	0.843	0.017	0.997
(21)		13	0.3–920	61	5.77	0.840	5.72	0.844	0.022	0.995
(22)	Dark	7	0.7–530	41	2.67	0.833	2.63	0.839	0.032	0.993
(23)		10	0.7–530	40	3.50	0.858	3.42	0.867	0.043	0.989
(24)		13	0.7–530	38	4.42	0.870	4.36	0.875	0.033	0.994
SERIES 2 EXPERIMENTS										
$\dot{V}_{O_2}$ (nmol ind.$^{-1}$ h^{-1}) vs dry mass (mg ind.$^{-1}$)										
(25)	F/light	10	1.0–350	42	149.36	0.818	148.93	0.820	0.015	0.997
(26)	F/dark	10	1.0–220	35	108.48	0.867	108.12	0.868	0.017	0.997
(27)	S/light	10	1.0–300	41	152.93	0.795	151.82	0.798	0.023	0.992
(28)	S/dark	10	1.0–500	43	119.45	0.815	118.87	0.817	0.018	0.995
$\dot{V}_{NH_3}$ (nmol ind.$^{-1}$ h^{-1}) vs dry mass (mg ind.$^{-1}$)										
(29)	S/light	10	1.0–350	41	17.10	0.893	16.93	0.898	0.030	0.990
(30)		10	1.0–220	35	10.98	0.992	10.84	0.997	0.037	0.989
(31)	S/dark	10	1.0–300	41	26.40	0.573	25.97	0.580	0.030	0.975
(32)		10	1.0–500	42	18.51	0.632	18.03	0.643	0.037	0.967
SERIES 3 EXPERIMENTS										
$\dot{V}O_2$ (nmol ind.$^{-1}$ h^{-1}) vs dry mass (mg ind.$^{-1}$)										
(33)	Light	5	0.04–0.07	69	90.65	0.990	88.58	1.648	0.332	0.339
$\dot{V}NH_3$ (nmol ind.$^{-1}$ h^{-1}) vs dry mass (mg ind.$^{-1}$)										
(34)	Light	5	0.04–0.07	64	42.35	1.380	478.15	2.210	0.438	0.390

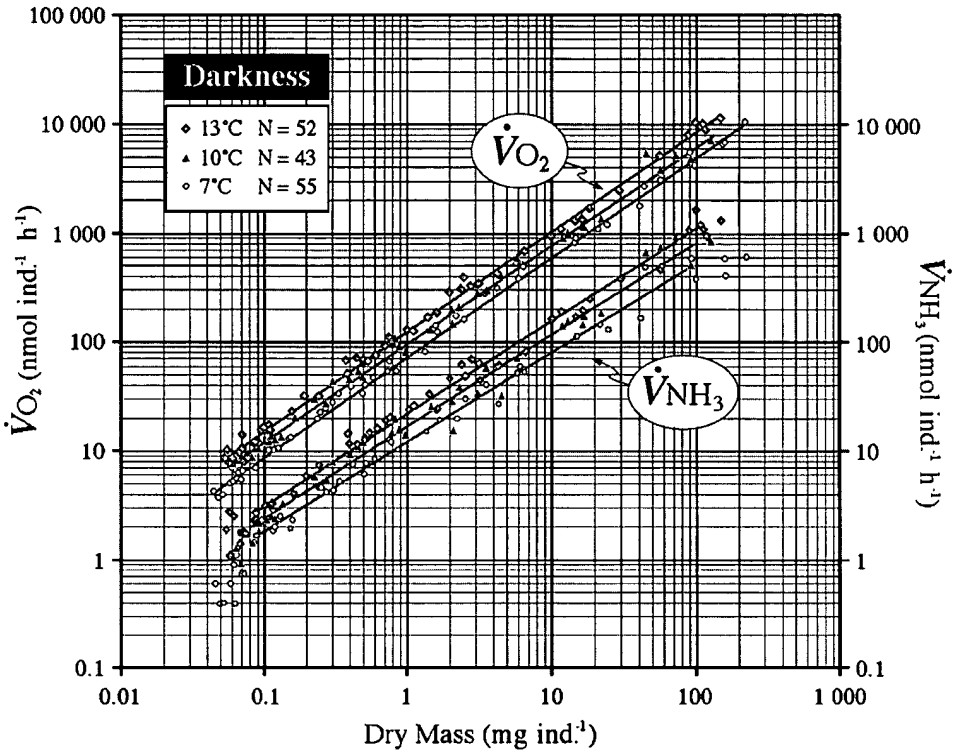

Fig. 5. *Gadus morhua*. Routine rate (nmol ind.$^{-1}$ h^{-1}) of oxygen uptake ($\dot{V}_{O_2}$) and ammonia excretion ($\dot{V}_{NH_3}$) related to dry mass of individual fasted larvae of Atlantic cod (Series 1) at 3 temperatures under conditions of darkness. Curves are modelled as described for Fig. 4

scaling (b = 1.380 ± 0.438), while Model II (v = 2.210 ± 0.438) was significantly higher than unity (Eq. 34; Table 2). The effect of light was similar to that described for $\dot{V}_{O_2}$, where smaller larvae were stimulated to excrete greater amounts of NH_3, and this effect declined with increasing size (data not shown). Modelling $\dot{V}_{NH_3}$ against wet mass and dry mass revealed that temperature affected $\dot{V}_{NH_3}$ with an average Q_{10} of 2.88 to 3.06 for light-adapted larvae and 2.32 to 2.61 for dark-adapted larvae.

The apparent NQ, calculated by comparing the molar ratios of $\dot{V}_{NH_3}$ and $\dot{V}_{O_2}$ of the light- and dark-adapted larvae, revealed that small larvae utilise greater amounts of amino acids as fuel than do larger larvae (Figs. 8 & 9). The data show that during the first phase of isometric growth (up to 7 mm SL) Atlantic cod larvae had high levels of amino-acid catabolism. If lipid was the dominant co-substrate, then amino-acid catabolism would have exceeded 70% of the total metabolic expenditure, and in some individuals amino acids may have constituted as much as 90 to 95% of fuel preference. No significant shift in fuel preference could be detected with increasing temperature in either light- or dark-adapted larvae; however, the dark-adapted larvae showed greater fluctuation in their NQs compared with light-adapted larvae. With continued growth and development, the NQ of both

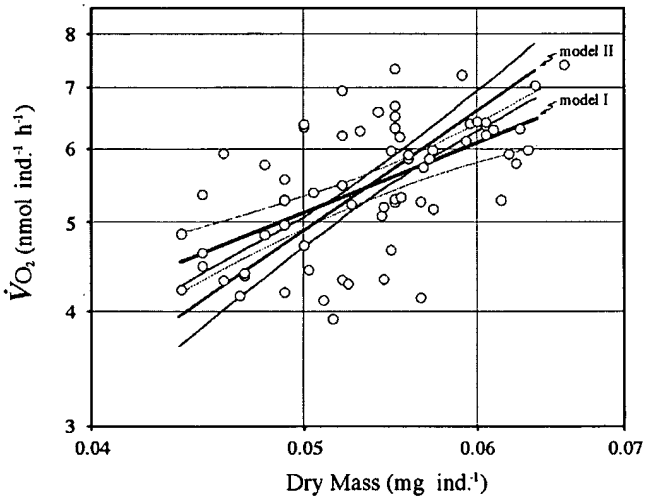

Fig. 6. *Gadus morhua*. Routine rate of oxygen uptake ($\dot{V}_{O_2}$, nmol ind.$^{-1}$ h^{-1}) related to dry mass of individual first-feeding-stage larvae of Atlantic cod (Series 3). The measurements were conducted at 5°C with larvae exposed to 10–25 µE s^{-1} m^{-2} at the respirometer surface. Each data point represents the mean $\dot{V}_{O_2}$ of 4–5 pooled larvae (N = 67 separate measurements). The larvae were derived from the simultaneous spawnings of 14 female Atlantic cod and were reared at 5°C to the completion of yolk resorption. The respiration measurements were conducted (Day 26 post-fertilisation, first-feeding stage) when the last remnants of yolk had disappeared

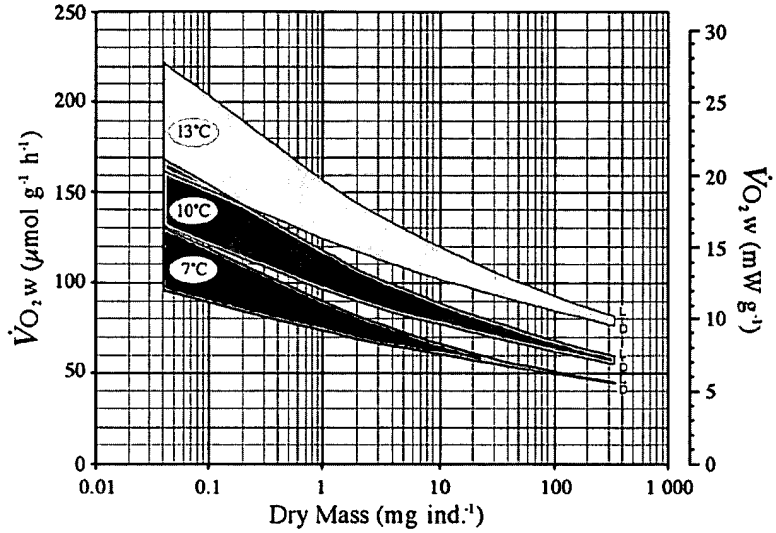

Fig. 7. *Gadus morhua*. Mass-specific routine rate of oxygen uptake ($\dot{V}_{O_2w}$, µmol g^{-1} h^{-1}) related to dry mass of individual larvae and early juvenile Atlantic cod exposed to light (10–25 µE s^{-1} m^{-2}) and darkness at 3 temperatures. The upper curves of each temperature region represents $\dot{V}_{O_2w}$ of light-adapted larvae (L), and the lower curves of each temperature region represents $\dot{V}_{O_2w}$ of dark-adapted larvae (D). The axis for the power rate of heat dissipation (mW g^{-1}) is constructed using an oxycaloric equivalent of 450 kJ mol^{-1} O_2 (Gnaiger & Kemp 1990). The circled numbers above the graph represent the 5 larvae depicted in Fig. 2

light- and dark-adapted individuals declined at each experimental temperature, and non-nitrogenous fuels became more important. However, even in juveniles (>12 mm SL) amino acids still constituted a substantial proportion of the total fuel budget, and, if lipids were the dominant co-substrate, would have supplied as much as 50 to 70 % of the fuel preference.

Comparison of the $\dot{V}_{O_2}$ of fed and short-term-fasted cod juveniles (Series 2) at 10°C did not reveal a significant increase in the aerobic metabolic rate associated with feeding of the light-adapted juveniles of any size (Fig. 10; Eqs. 25 & 27; Table 2). The same was true for dark-adapted juveniles up to a dry mass of 10 mg ind.$^{-1}$, although the regression analyses over the full range of sizes examined revealed a small, but significantly ($p < 0.05$) lower, slope of the fasted juveniles (Fig. 11; Eqs. 26 & 28; Table 2). Conversely, the drop in $\dot{V}_{NH_3}$ of short-term-fasted juveniles with dry masses above 60 mg ind.$^{-1}$ (>35 mm SL) was highly significant, as reflected in the low regression coefficients of the short-term-fasted juveniles compared to the fed juveniles (Eqs. 29–32; Table 2). For juveniles less than 10 mg ind.$^{-1}$ dry mass (<20 mm SL), no significant depression of $\dot{V}_{NH_3}$ was observed. The consequence of the significantly lower $\dot{V}_{NH_3}$, while maintaining near-fed rates of $\dot{V}_{O_2}$, is clearly seen when examining the *NQ*s (Fig. 12). Short-term fasting did not influence the fuel preference of juveniles with SLs of less than 20 mm, but may have caused a significant switch to non-nitrogenous fuels in juveniles with SLs in excess of 30 mm. The presence or absence of light had no effect in this respect. Compared with the *NQ*s of the short-term-fasted juveniles of Series 1, the *NQ*s of Series-2 juveniles were lower.

DISCUSSION

There is considerable debate in the literature as to which regression model is most appropriate for the unbiased analysis of bivariate biological data (Ricker 1973, Laws & Archie 1981, Giguère et al. 1988, Packard & Boardman 1988, LaBarbera 1989, Post & Lee 1996, Clarke & Johnston 1999, Sokal & Rohlf 2000). Statisticians argue unanimously that Model II (geometric-mean) regression yields the unbiased estimate of *y* as a function of *x*, and

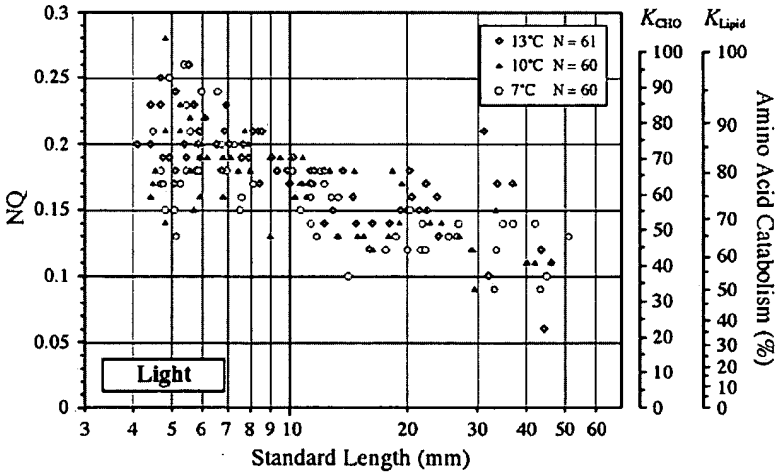

Fig. 8. *Gadus morhua*. Apparent nitrogen quotient (*NQ*) related to SL of larvae and early juvenile Atlantic cod at 3 temperatures exposed to light (10–25 µE s^{-1} m^{-2} at the respirometer surface). Depending on which co-substrate (carbohydrate [K_{CHO}] or lipid [K_{lipid}]) is catabolised, the *NQ* yields an estimate of the relative contribution of amino acids as fuel. The 2 axes ([K_{CHO}] and [K_{lipid}]), denoting the relative fraction of amino-acid catabolism, were calculated following the recommendations of Gnaiger (1983). Note that, when lipid is the co-substrate catabilised, the relationship between the *NQ* and the mass fraction of amino acids burned is non-linear

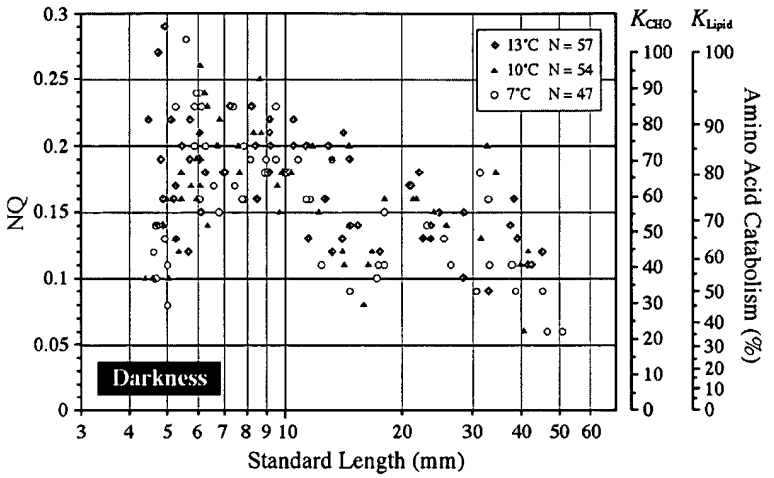

Fig. 9. *Gadus morhua*. Apparent nitrogen quotient (*NQ*) related to SL of larvae and early juvenile Atlantic cod at 3 temperatures under conditions of darkness. The fuel axes were constructed as described for Fig. 8

x as a function of *y*. Since both data variates (i.e. respiration rate and body mass) were not controlled by the investigator, but vary independently, then Model-II techniques should be applied (Ricker 1973, Laws & Archie 1981, Giguère et al. 1988, LaBarbera 1989, Sokal & Rohlf 2000). In the literature, however, the great majority of studies have used the least-squares linear approach (Model I) to describe scaling relationships, usually arguing that the measurement of body mass can be achieved with a minimum of error due to the precision of modern microbalances. This seems a reasonable approach, despite the fact that the statistical literature does not accept this thesis. Model-I regression, however, has the major disadvantage of being highly sensitive to outliers, since their influence is proportional to the square of their distance from the mean. As a result any slope derived from Model-I regression analysis is notoriously influenced by data points at either end of the scale. The debate of the most appropriate regression model is very much an ongoing one (Clarke & Johnston 1999), and there is no simple answer. In part, to avoid the controversy, but also to ensure that our interpretations are not shackled by structural modelling, we chose to present both models.

Although the presented data are very low in variance and yield almost identical equations, regardless of the regression model applied, whenever the scale of the abcissa was reduced, the variance of the data was too great to arrive at some reasonable estimate of the exponent (Eqs. 33 & 34; Table 2). The finding that the metabolic exponent of the Series-3 experiments (14 groups of first-feeding larvae) did not differ significantly from unity does not necessarily mean that the metabolic rate of Atlantic cod larvae scales isometrically with body mass. We consider the abscissal scale to be insufficient to arrive at such a conclusion. The overwhelming empirical evidence of the $\dot{V}_{O_2}$ of Series-1 and Series-2 experiments, regardless of temperature or exposure to light, argues strongly that the routine metabolic rate of Atlantic cod larvae and early juveniles does not scale isometrically with body mass. Indeed, an allometric scaling of metabolic rate with body size in Atlantic cod is unambiguously supported in the literature, regardless of stage investigated (Sundnes 1957, Saunders 1963, Edwards et al. 1972, Laurence 1978, Serigstad 1987a).

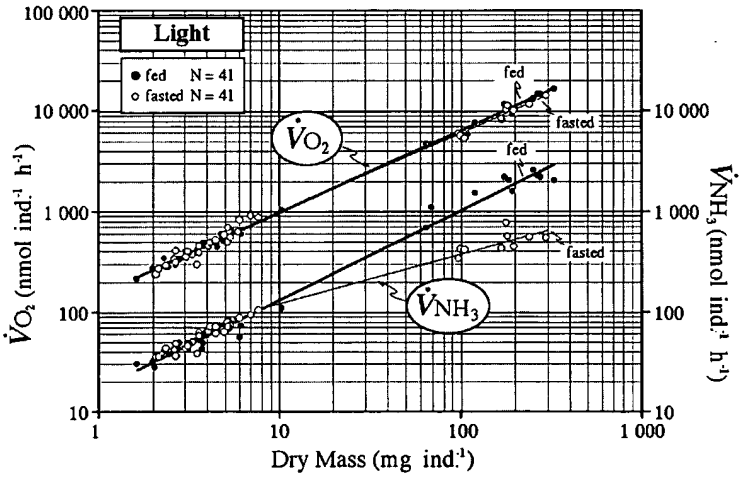

Fig. 10. *Gadus morhua*. Rate (nmol ind.$^{-1}$ h^{-1}) of oxygen uptake ($\dot{V}_{O_2}$) and ammonia excretion ($\dot{V}_{NH_3}$) related to dry mass of individual fed and fasted larvae and early juvenile Atlantic cod (Series 2) at 10°C exposed to 10–25 µE s^{-1} m^{-2} at the respirometer surface. Curves are modelled as described for Fig. 4

The current findings are thus at odds with the hypothesis of Giguère et al. (1988), who believed that metabolic rates scale isometrically in larval fishes, and also with the proposal of Bochdansky & Legget (2001), who argue that metabolic scaling during ontogeny is curvilinear. The present routine metabolic data also do not fit the first isometric or near-isometric phase deduced by Post & Lee (1996). These earlier studies did not provide any fundamental reason other than careful experimentation and statistical analyses for their general assessments. There is little doubt

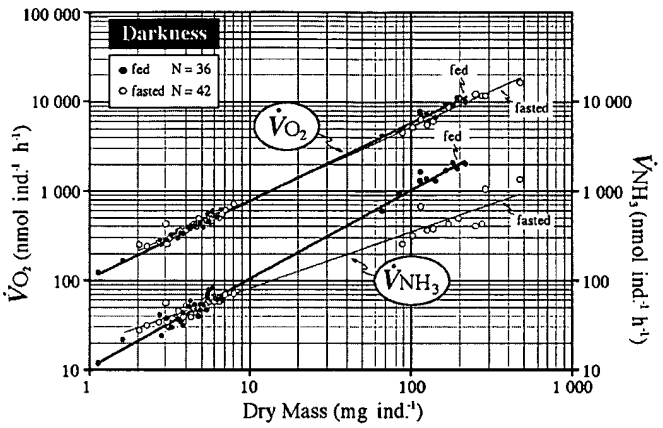

Fig. 11. *Gadus morhua*. Rate (nmol ind.$^{-1}$ h^{-1}) of oxygen uptake ($\dot{V}_{O_2}$) and ammonia excretion ($\dot{V}_{NH_3}$) related to dry mass of individual fed and fasted larvae and early juvenile Atlantic cod (Series 2) at 10°C under conditions of darkness. Curves are modelled as described for Fig. 4

that some larval fishes scale isometrically with body size (e.g. Atlantic mackerel *Scomber scombrus*, Giguère et al. 1988; Chinook salmon *Oncorhynchus tshawitscha*, Rombough 1994; and Atlantic halibut *Hippoglossus hippoglossus*, Rønnestad & Naas 1993, Finn et al. 1995d), but it is clear that the generalisation for all fish larvae is not valid. This agrees with the views of Wieser (1995). We are therefore faced with trying to explain the fact that Atlantic cod larvae, even at the very earliest stages of their life cycle, do not have metabolic rates that are proportional to their body size. The allometric metabolic exponent is maintained throughout the life of Atlantic cod and does not equate to the general larval isometry (Giguère et al. 1988), nor to the 3/4-power scaling coefficient first noted by Kleiber (1932). In recent years, several articles have argued that it is the fractal vascular networks of plants and animals which limit the supply of nutrients to cells in such a way as to dictate a quarter-power scaling law (West et al. 1997, 1999, 2001, 2002, Banavar et al. 1999, Enquist et al. 1999). Indeed, Bochdansky & Leggett (2001) used the vascular argument modulated by the surface law as the basis of one of their structural terms and the isometric argument of Giguère et al. (1988) for the other term. Others have argued that the relative importance of branchial or cutaneous gas exchange may be the cause of the observed scaling coefficient (Hughes 1984, Hughes & Al-Kadhomiy 1988). More recently, Oikawa & Itazawa (1993) and Wells & Pinder (1996a,b) have concluded that there is little evidence that respiration in larval fishes becomes limited by respiratory surface area, and Rombough & Moroz (1997) and Rombough (1998) have argued that, despite dealing with gas exchange, the primary function of gills in fish larvae is more related to ionoregulation. We agree with these latter ideas, and thus argue that some other reason must be found for the observed metabolic exponents of 0.88 and 0.91 in the early life stages of Atlantic cod (Series 1) in light and darkness, respectively.

The 2 bivariate parameters that are measured are routine $\dot{V}_{O_2}$ and body mass. Routine $\dot{V}_{O_2}$ as discussed by Fry (1957) and Beamish & Dickie (1967) includes all metabolic levels between standard and active rates, and it applies to measurements conducted on spontaneously active larvae. The point here is that the routine metabolic rate is one that is not forced, i.e. oxygen delivery is not limited, and cellular metabolism is not stressed to its maximum, but lies at the lower end of the metabolic scope. Since the actual site of oxygen consumption occurs at the end of the electron-transport system within the mitochondrial matrix, it seems logical that the metabolic rate might in some way be related to the amount of mitochondria in the organism. Using histological and stereological techniques, Galloway et al. (1998) found that the axial swimming musculature is the largest and most rapidly growing tissue in the larval body. Galloway et al. (1999) also report, albeit for a limited size range, that the white-muscle fractions grow at faster rates than the red muscle in Atlantic cod larvae. The white fibres in Atlantic cod larvae constituted approximately 80% of the muscle cross-sectional area at first feeding (4 mm SL) and 90% at 7.5 mm SL. This rep-

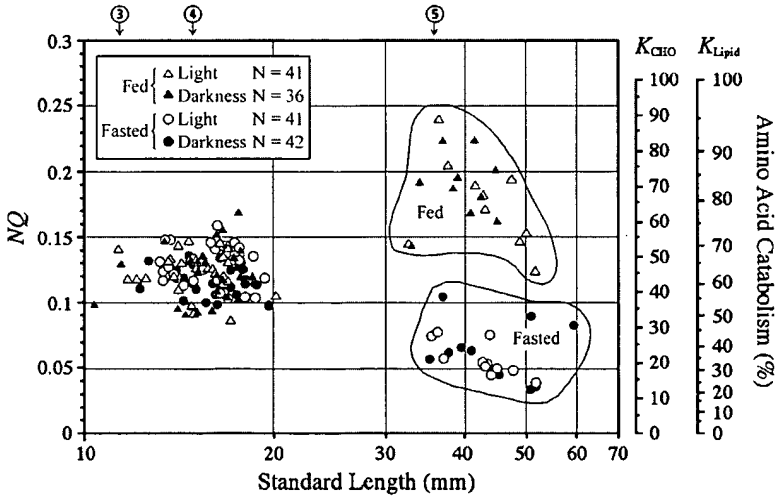

Fig. 12. *Gadus morhua*. Apparent nitrogen quotient (*NQ*) related to SL of larvae and early juvenile Atlantic cod at 10°C under conditions of darkness. The fuel axes were constructed as described for Fig. 8

resents the isometric growth phase of the larvae, when body mass is increasing to the cube power of SL (Table 1). The density of mitochondria in the red- and white-muscle fibres of Atlantic cod larvae was also found to change with development, such that the fraction of red-muscle mitochondria increases while that of the white muscle decreases (T. F. Galloway pers. comm.). The suggestion is therefore that there is an ontogenetic reorganisation of tissues which form part of the body mass but which contribute less to the $\dot{V}_{O_2}$, and, as a result, aerobic metabolic rate scales allometrically with size even in the earliest stages of Atlantic cod larvae. The reorganisation for Atlantic cod is suggested to be primarily the allometric development of white muscle, but could easily be other forms of ametabolic tissues (e.g. the labile energy stores of leptocephalus larvae) and as such will be species or even race specific, as indicated by the many separate regression lines plotted by Post & Lee (1996). Such a tissue-specific basis for the mass-metabolic exponent has already been discussed (Itazawa & Oikawa 1983, Oikawa & Itazawa 1983, 1985, 1993, Wieser 1995) and most recently argued by Darveau et al. (2002). An exaggerated example of allometric metabolic scaling is seen in the leptocephalus larvae studied by Pfeiler & Govoni (1993) and Bishop & Torres (1999). Leptocephalus larvae have the remarkable adaptation of accumulating ametabolic energy stores in the form of proteoglycans and glycosaminoglycan carbohydrates (Pfeiler 1996, Bishop et al. 2000) which contribute to the body mass but not to the metabolic rate.

The present data for Atlantic cod larvae clearly demonstrate that, given unlimited food supplies, temperature, light and body size are the principle extrinsic and intrinsic factors governing the metabolic rate. Previous studies have demonstrated that temperature and size are the greatest determinants of growth in Norwegian coastal and northeast Arctic Atlantic cod larvae (Otterlei et al. 1999, Otterlei 2000). These earlier studies also noted that instantaneous growth rates can exceed 25% d^{-1}, as found in the present study. Otterlei et al. (1999) found that maximum growth rates of Atlantic cod occur in larvae of less than 1 mg $ind.^{-1}$ dry mass. If we assumed, as a worst-case scenario, that 75% of the smallest larvae in the present study either died or were eaten, then instantaneous growth rates would still have exceeded 25% d^{-1}. Thus, although we cannot rule out the possibility of cannibalism or size-selective mortality in the mesocosms, the present data indicate that such high growth rates may be maintained beyond metamorphosis.

During the 48 d study period of Series 1, the increase in larval body mass spanned 5 orders of magnitude. According to Scott & Scott (1988, cited by Fahay et al. 1999) the largest Atlantic cod ever recorded was 95.9 kg, which means that during the life of Atlantic cod body mass spans 9 orders of magnitude. To achieve the ninth order of magnitude, however, cod will need to grow for perhaps another 30 to 40 yr (11 000 to 14 600 d). The comparison emphasises that one of the main missions of fish larvae during their early life is to grow. A second point worth noting is that during these 48 d of growth, the Atlantic cod larvae increased their size in the same scale as a mouse to a horse. Had we continued our studies for another 2 wk, the cod larvae would have scaled from mouse to elephant, but the metabolic exponent of $\dot{V}_{O_2}$ as a function of body mass was significantly higher than the 0.75 noted for the well-known mouse-to-elephant curve (Eckert 1997). As noted by others (Heusner 1982, Feldman & McMahon 1983, Riisgård 1998) even a small difference in the scaling exponent has profound effects over several orders of magnitude. Indeed, if the metabolic exponent of $\dot{V}_{O_2}$ versus dry body mass had been 0.76 (i.e. different only at the second decimal), then, even over the 5 orders of magnitude found, $\dot{V}_{O_2}$ would have been 6% higher in the largest larvae (350 mg $ind.^{-1}$ dry mass) compared with those modelled with an exponent of 0.75. The observation that the metabolic exponent was actually 0.88 to 0.91 means that $\dot{V}_{O_2}$ in the largest larvae was between 115 and 150% higher than for an equivalently sized larva that had scaled according to the 3/4-power law.

The effect of acute acclimation to temperature of between 7 and 13°C, with additional measurements at 5°C, shows that metabolic scaling of the early life stages of Atlantic cod is not affected by altered environmental temperature. Rather, the metabolic rate shifts up or down in an exponential relation to the increase or decrease in temperature. These findings are supported by the studies of Giguère et al. (1988) for larval mackerel. Modelling the aerobic metabolic rate of light-adapted larvae of Atlantic cod at first-feeding (dry mass = 0.06 mg $ind.^{-1}$) measured at 5, 7, 10 and 13°C in the present study revealed an almost perfect exponential relationship between routine $\dot{V}_{O_2}$ and temperature, T ($\dot{V}_{O_2} = 63.349e^{0.094T}$; $r^2 = 0.9999$). The exponent 0.094 of the light-adapted larvae is unaffected by size, and hence, using the same procedure for the early life stages in darkness, we can derive 2 general equations describing the aerobic metabolic rate of Atlantic cod:

$$\dot{V}_{O_2}\ (\text{nmol ind.}^{-1}\ \text{h}^{-1})\ \text{in light} = 45.824\ \text{dry mass}^{0.8851}\ e^{(0.094T)}$$

$$\dot{V}_{O_2}\ (\text{nmol ind.}^{-1}\ \text{h}^{-1})\ \text{in darkness} = 39.879\ \text{dry mass}^{0.9124}\ e^{(0.0877T)}$$

where dry mass is in mg $ind.^{-1}$ and T in °C. For estimation of the power rate of heat dissipation in μW $ind.^{-1}$,

the product of the above equations can be multiplied by 0.125. Similary for the $\dot{V}_{NH_3}$ the 2 equations are

$$\dot{V}_{NH_3} \text{ (nmol ind.}^{-1}\text{ h}^{-1}\text{) in light} = 6.761 \text{ dry mass}^{0.8118}\, e^{(0.1075T)}$$

$$\dot{V}_{NH_3} \text{ (nmol ind.}^{-1}\text{ h}^{-1}\text{) in darkness} = 6.351 \text{ dry mass}^{0.8337}\, e^{(0.0957T)}$$

Such general equations are of the form given by Giguère et al. (1988) and most recently promoted by Gillooly et al. (2001, 2002), who argued that models based on Q_{10} coefficients can result in errors of up to 15%.

The classical Q_{10} estimates of the effect of temperature on routine metabolic rate do, however, provide a conveniently recognisable scale for the thermal sensitivity of reaction rates, and they were therefore calculated for mean metabolic exponents of light- and dark-adapted larvae. Since temperature did not significantly affect the metabolic exponents of either light- or dark-adapted individuals, there was no statistical basis for suggesting a size-related effect of temperature on the metabolic rate of larval and early juvenile Atlantic cod. Hence we find that between 7 and 13°C dark-adapted larvae have a mean Q_{10} of 2.4, while light-adapted larvae have a mean Q_{10} of 2.6 for the size ranges studied. These quotients will also apply to light-adapted larvae down to 5°C. Our findings are therefore in good agreement with those reviewed by Rombough (1988) and Clarke & Johnston (1999) for within species thermal sensitivity, and they support the earlier notion that teleost red-muscle mitochondria have only evolved modest adjustments for temperature adaptation (Johnston et al. 1994).

Marine fish larvae are quite capable of utilising glycolytic pathways for substrate phosphorylation, but these pathways are overshadowed by the activities of the tri-carboxylic acid cycle enzymes (Wieser 1991, Segner et al. 1994, Overnell & Batty 2000). This is hardly surprising considering that the majority of fish larvae are carnivorous and adapted to deal with the amino-acid surge associated with the digestion of prey organisms. Since lipids are preferentially catabolised in juvenile and adult fishes (Wood 2001), we assume that lipids are also the co-substrate for interpreting the apparent *NQ* data for Atlantic cod larvae. Indeed, both polar and neutral lipids were found to comprise the major co-substrate of Atlantic cod during yolk resorption (Finn et al. 1995a,b).

Although most nitrogen is excreted as ammonia in fish larvae (Wright & Fyhn 2001), recent studies of marine fish larvae, including Atlantic cod, indicate that urea can be a significant form of waste nitrogen (Chadwick & Wright 1999, Terjesen et al. 2002). These findings are in contrast to the situation for adult Atlantic cod, where activities of urea cycle enzymes are low (Chadwick & Wright 1999). Nevertheless, if significant amounts of urea derived from deamination of ingested amino acids do occur, then the apparent *NQ*s are minimum estimates of the mass fraction of amino acids utilised as fuel. Any ammonia production from adenylate deamination resulting from burst swimming events will not affect this interpretation, since most ammonia is retained intracellularly (Wood 2001).

Few studies have examined the effect of feeding on $\dot{V}_{O_2}$ in fish larvae, although an increased thermogenesis associated with feeding is well known in juveniles and adults (Jobling 1981, 1994, Blaikie & Kerr 1996). For marine fish larvae, Giguère et al. (1988) found an increased metabolic rate in fed mackerel larvae, as did Torres et al. (1996) in fed larvae of red drum *Sciaenops ocellatus*. The major difference between the findings of these studies was that Giguère et al. (1988) did not observe any effect of food on metabolic scaling, while Torres et al. (1996), based on considerably less data, suggested that the presence of food can elevate the metabolic exponent. The lack of difference in the $\dot{V}_{O_2}$ of fed versus short-term-fasted Atlantic cod larvae in the present study may in part be due to the 1 h acclimation period prior to experimentation or to increased spontaneous activity associated with hunger in the short-term-fasted group.

Regarding the metabolic fuel of the larvae in the present study, no difference in the *NQ* was seen between fed and short-term-fasted larvae of less than 20 mm SL (~10 mg ind.$^{-1}$ dry mass). In larvae greater than 30 mm SL (~40 mg ind.$^{-1}$ dry mass) a substantial decrease in the $\dot{V}_{NH_3}$ compared to the $\dot{V}_{O_2}$ was observed in the short-term-fasted larvae, suggesting a major switch in the fuel preference. Since adult fishes do not show altered daily rates of urea excretion even in the presence or absence of food (Wood 2001), we suggest that short-term-fasted juveniles of Atlantic cod are capable of conserving amino acids, rather than funneling them into the tri-carboxylic acid cycle.

In conclusion we find that the routine metabolic rate of Atlantic cod larvae scales allometrically with body mass. This functional relationship is not altered by increased temperature. Light causes a 1.4-fold elevation in routine metabolic rate of first-feeding larvae, but becomes less influential with increasing size, until no effect is seen in metamorphosed juveniles. Increasing acclimation temperature results in Q_{10} effects of 2.4 and 2.6 in dark- and light-adapted larvae, respectively. Atlantic cod larvae show a high preference for amino acids as their major fuel during the first 3 to 4 wk of post-hatch development. Juveniles seem capable of switching fuels during short-term fasting events, and thereby conserve amino acids.

Acknowledgements. We are grateful to Marianne Meling for assistance with the respiration studies of the cod larvae at 5°C, and we are indebted to Trina Galloway for furnishing mitochondrial density data of Atlantic cod red and white axial muscle. Per Solemdal kindly provided the Series-3 cod eggs. The study was supported by the Norwegian Research Council (grants 121695/122, 141990/120), and is contribution no. 114 of the Marine Larvae Locus at the University of Bergen.

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Submitted: February 26, 2002; Accepted: June 21, 2002
Proofs received from author(s): October 16, 2002

Attachment B

ENVIRONMENTAL ANALYSIS

Determination of Carbon Dioxide Evolution Rates Using a Novel Noninstrumental Microrespirometer

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A novel noninstrumental microrespirometer was developed to determine carbon dioxide evolution rates of solid or liquid samples at μL/h levels accurately and rapidly. The respirometer is based on the simple principle of acid–base titration at a steady-state of carbon dioxide absorption/evolution. The structure and operation of the microrespirometer are simple and the cost is modest in comparison to instrumental methods. The microrespirometer is suitable for laboratory studies and field routine examinations of food, agricultural, and environmental samples.

Respiration is a common indicator of biological activity. Respirometry (measurement of respiration rates) has been applied to a broad spectrum of applied and environmental microbiology, such as toxicity–treatability, process control and prediction of biological oxygen demand (BOD_5) in wastewater treatments (1, 2), assessment of metal toxicity (3), living soil microbial biomass (4–6), and food quality (7, 8).

Respiration rates can be measured either by rates of oxygen consumption or CO_2 evolution. Rapid oxygen consumption rate can be measured by using an oxygen probe or a quantitative electrolytic cell (2). Most oxygen respirometers, however, are applicable only to liquid samples. Oxygen respirometers with an electrolytic cell can be used to determine respiration of solid or semi-solid samples, but their sensitivity is greatly compromised.

Sensitive and rapid CO_2 respirometers based on infrared (IR) detectors have been developed in the last 3 decades (9), and can handle solid samples with high speed and sensitivity. Instrumental respirometers are technically complicated and expensive if accuracy and sensitivity are demanded. Noninstrumental CO_2 respirometers operated by an alkaline trap and acid–base titration have been in existence for years (10). They are simple but relatively slow (measurement in days) and less sensitive (detection limit in mL CO_2/day). Sensitive and rapid determination of respiration rates is highly desirable in monitoring microbial activity in food and environmental samples.

We developed a novel non-instrumental microrespirometer capable of determining CO_2 evolution rates of solid or liquid samples rapidly (in 1 h) and sensitively (μL/h level). The operation is simple and the cost is very modest. The microrespirometer can be conveniently operated under laboratory or field conditions.

Received July 19, 1999. Accepted by JS November 2, 1999.
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Experimental

Materials

(a) *Microrespirometer.*—The design of the microrespirometer is based on the simple principle of acid–base titration between an alkaline solution and CO_2, with phenolphthalein to indicate the end point. The microresprometer consists of a transparent reaction chamber and a sample vial (Figure 1). The reaction chamber is, in fact, a small alkaline trap (total headspace, 6–7 mL) with a septum hole. The size of a sample vial may vary depending on the need (vials of 25, 30, 40, and 75 mL are available; e.g., Fisherbrand [Suwanee, GA] EPA bottles). The reaction chamber and the sample vial are coupled through a standard threaded screw and septum liner to form a closed headspace. Alkaline solution can be injected into the reaction chamber with a syringe. The alkaline solution absorbs the CO_2 in the headspace. The indicator in the alkaline solution changes from a deep to a faint pink when the alkaline solution is consumed by CO_2. The microrespirometer is shaken at a fixed rate of ca 240 rpm on an orbital shaker to enhance the absorption of CO_2.

(b) *Alkaline solution.*—The alkaline NaOH–$BaCl_2$–indicator solution contains an equal molar ratio of NaOH and $BaCl_2$ and 0.5 mL indicator solution (0.5% phenolphthalein in 50% ethanol solution) per 50 mL alkaline solution. $BaCl_2$ in the alkaline solution precipitates the absorbed CO_2 that ensures the stoichiometry of 2 moles of alkaline spent per mole of CO_2 absorbed, i.e.,

$$CO_2 + 2NaOH + BaCl_2 = BaCO_3(s) + 2NaCl \quad (1)$$

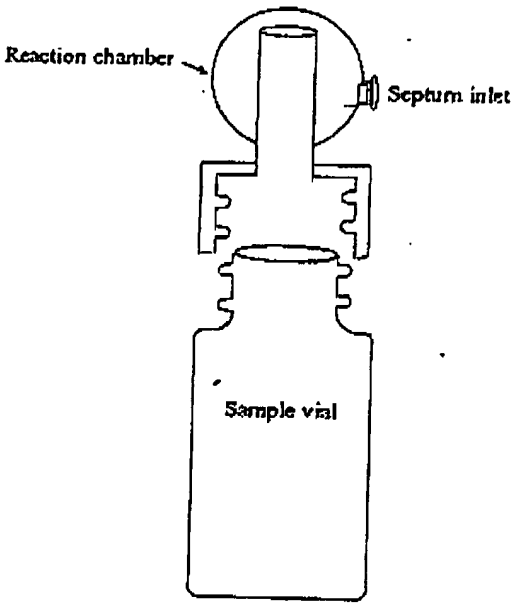

Figure 1. A graphic representation of the microrespirometer. The reaction chamber has a total headspace of 6–7 mL in which up to 1.2 mL can be used to hold the alkaline indicator solution. The size of the sample vial can be varied according to the test. Currently, 25, 40, and 75 mL sample vials are available commercially.

$BaCl_2$ also sharpens the change of color at the end point when a very low level of respiration is being determined. The alkaline solution is stored in a septum-capped vial to prevent absorption of CO_2 from the air. The alkaline solution is transferred through a syringe.

Procedures

The following experiments were performed to determine the optimal conditions for operating the microrespirometer:

(a) *Shaking rate experiment.*—The effect of shaking on the CO_2 absorption of the microrespirometer was investigated. Microrespirometers with empty 25 mL sample vials were coupled in a glove box of known CO_2 concentration (determined by an IR CO_2 analyzer). A 0.2 mL portion of 0.002M alkaline solution was injected into each reaction chamber. The microrespirometers were shaken at a fixed rate of 100, 150, 200, 250, or 300 rpm. The time required to consume the alkaline solution in each microrespirometer (as indicated by the color change) was recorded. Each treatment was performed in triplicate.

(b) *Alkaline concentration experiment.*—The effect of alkaline concentration on the absorption of CO_2 in a closed headspace was investigated. A 25 mL sample vial was connected to an IR analyzer as described by Hsieh and Hsieh (9) so that the vial and the IR detector formed a closed headspace in which air circulated continuously. The 25 mL vial was shaken at 240 rpm on an orbital shaker. A 1 mL portion of 0.2, 0.1, 0.01, or 0.001M alkaline solution was injected into the vial through a syringe needle port at the beginning of the experiment and the concentration of CO_2 in the vial was recorded periodically. The experiment was repeated twice.

(c) *CO_2 concentration experiment.*—The relationship between the CO_2 absorption rate and the CO_2 concentration in the headspace of the microrespirometer was investigated. Microrespirometers with a 75 mL sample vial were coupled in a glove box of known CO_2 concentration. An increment of 0.1 mL 0.002M alkaline solution was injected into the reaction chamber. The microrespirometers were shaken at 240 rpm and the time required to consume each increment of the alkaline solution was recorded. The consumption of each increment of the alkaline solution (i.e., 0.2 μmol alkaline, or 0.1 μmol CO_2) represents a 29.7 ppm (v/v) reduction of CO_2 concentration in the 82 mL microrespirometer at 25°C. Each treatment was performed in triplicate.

(d) *Microrespirometer procedure.*—A portion of solid or liquid sample was place in a sample vial and the vial was coupled to a reaction chamber. 0.8 mL alkaline solution of suitable concentration was injected into the reaction chamber using a syringe. The respirometer was shaken at a fixed rate of ca 240 rpm for 30 min (the pre-equilibration period), ensuring that the alkaline solution was not completely consumed during this time. If the alkaline solution wass about to be consumed, more alkaline solution was injected into the reaction chamber. After 30 min pre-equilibration, the shaker was stopped and the alkaline solution in the reaction chamber was

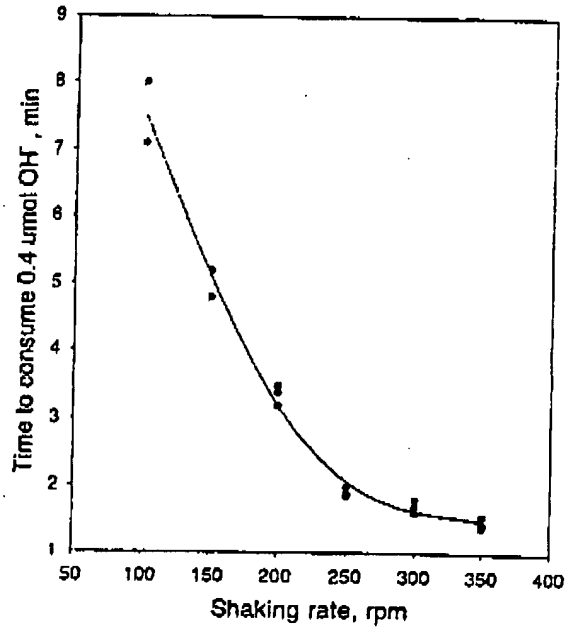

Figure 2. Relationship between CO_2 absorption and shaking rate of the microrespirometer at 25°C. Each dot represents a single measurement, not a mean.

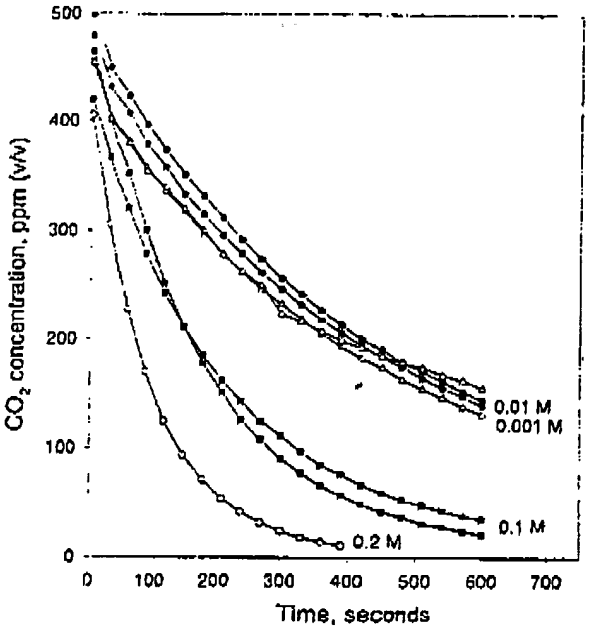

Figure 3. Relationship between CO_2 absorption and concentration of the alkaline solution in the microrespirometer. The experiment was performed at 25°C and a shaking rate of 240 rpm. Each dot represents a single measurement, not a mean.

withdrawn to ca 0.1–0.2 mL remaining. The respirometer continued to be shaken until the alkaline solution changed to a faint pink. The shaker was stopped immediately,and 0.1 mL alkaline solution was injected, shaking resumed, and the time required to consume the alkalinity was recorded. Increments of 0.1 mL alkaline solution were injected twice more and the time required to consume each increment was recorded. The average of the time required to consume the 0.1 mL increment alkaline solution was used to calculate the CO_2 evolution rate with the following formula:

$$CO_2 \text{ evolution rate, } \mu\text{mol/h} = \frac{0.1 \times 10^3 \times M/2}{t/60} \quad (2)$$

where M is the concentration of the alkaline solution in mol/L and t is the time required to consume 0.1 mL alkaline solution in min. The CO_2 evolution rate can be expressed in µL/h by multiplying the molar volume of CO_2 at a specific temperature.

(e) *Validation experiment.*—The CO_2 evolution rates determined by the microrespirometer method were compared with those determined by an established IR analyzer method. Portions of soil samples of relatively low CO_2 evolution rates (2–5 µL/h/g), unfrozen processed meat samples of medium CO_2 evolution rates (10–100 µL/h/5 g), and room temperature milk samples of high CO_2 evolution rate (80–280 µL/h/20 mL) were placed in 25 mL sample vials. The CO_2 evolved by microorganisms associated with each sample was determined by the microrespirometer method. A duplicate sample in another 25 mL sample vial was also placed in a 250 mL flask and the CO_2 evolution rate was determined by the IR analyzer method described by Hsieh and Hsieh (9). The sample vials in the microrespirometers and those in the 250 mL flasks of the IR analyzer were exchanged and the CO_2 rates were determined again with the alternative methods.

Results and Discussion

The effect of shaking rates on CO_2 absorption of the microrespirometer is shown in Figure 2. The CO_2 absorption increased as the shaking rate increased from 100 to 250 rpm. The increase in CO_2 absorption leveled off when the shaking rate exceeded 250 rpm. Shaking at 200 rpm or higher improved reproducibility of CO_2 absorption. A fixed shaking rate between 200 and 250 rpm is recommended for the microrespirometer because the benefit of shaking is achieved while the difficulty of operation at higher rates is avoided.

The results of the alkaline concentration experiment are presented in Figure 3. As the concentration of alkaline solution decreased from 0.2 to 0.01M, the CO_2 absorption rate decreased as well. The CO_2 absorption rate did not further decrease as the alkaline concentration was reduced from 0.01 to 0.001M. It is not possible to have complete absorption of CO_2 in the headspace of a microrespirometer in a matter of hours when the concentration of the alkaline solution is less than 0.01M. The concentration of the alkaline solution has to be

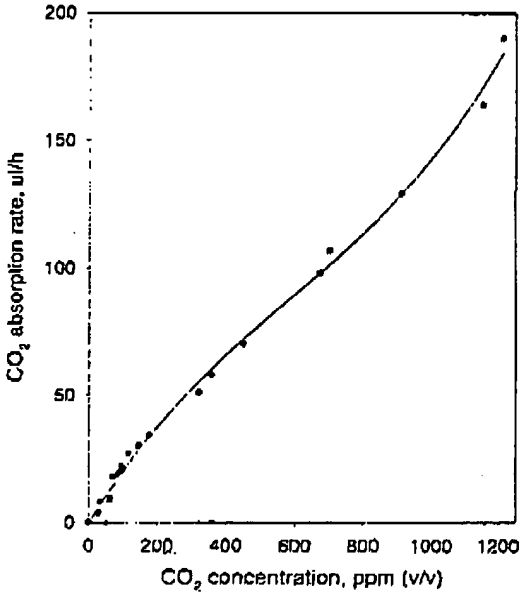

Figure 4. Relationship between CO_2 absorption and concentration of CO_2 in the headspace (82 mL) of the microrespirometer. The experiment was performed at 25°C and a shaking rate of 240 rpm. Each dot represents a single measurement, not a mean.

much less than 0.01M in order to determine CO_2 evolution rate at a μL/h level. The microrespirometer, therefore, does not work on the principle of a complete CO_2 absorption but on an absorption/evolution equilibrium principle that will be discussed in detail later. An alkaline solution of less than 0.0005M is not stable enough to be used in the microrespirometer because the possibility of contamination from the ambient CO_2 is too large for such low alkalinity. Phenolphthalein is not stable in alkaline concentrations exceeding 0.01M; the deep pink color will fade away by itself within 1 h. Therefore, a proper alkaline concentration range suitable for the microrespirometer is between 0.01 and 0.001M.

Figure 4 depicts the relationships between the CO_2 absorption rate of a 0.002M alkaline solution and the concentration of the CO_2 in the headspace of a microrespirometer. In general, the CO_2 absorption rate has a positive curve–linear relationship with the concentration of CO_2. The CO_2 absorption rate of the respirometer at a given temperature and shaking rate reflects the CO_2 concentration in the headspace of the microrespirometer which is, of course, not necessarily the CO_2 evolution rate of the sample. However, if a sample is equilibrated with the alkaline solution in the respirometer at a given temperature and a given shaking rate, then the concentration of CO_2 in the respirometer would eventually reach a constant value when the CO_2 absorption rate equals the CO_2 evolution rate. For example, if the beginning CO_2 concentration of the respirometer is 450 ppm and the CO_2 evolution rate of the sample is 100 μL/h, the CO_2 concentration of the respirometer will be increased to about 660 ppm (Figure 4) and stay there because an equilibrium of CO_2 absorption and evolution is established. If the CO_2 evolution rate of the sample is 20 μL/h, the CO_2 concentration of the respirometer will be decreased to about 150 ppm (Figure 4), where an absorption/evolution equilibrium is established. The CO_2 evolution rate of a sample, therefore, can be determined by the CO_2 absorption rate of the microrespirometer when an equilibrium or steady-state is established. That is, after a sample is equilibrated with an alkaline solution in a microrespirometer, the CO_2 evolution rate can be determined by the time required to consume a small increment of the alkaline solution (equation 2).

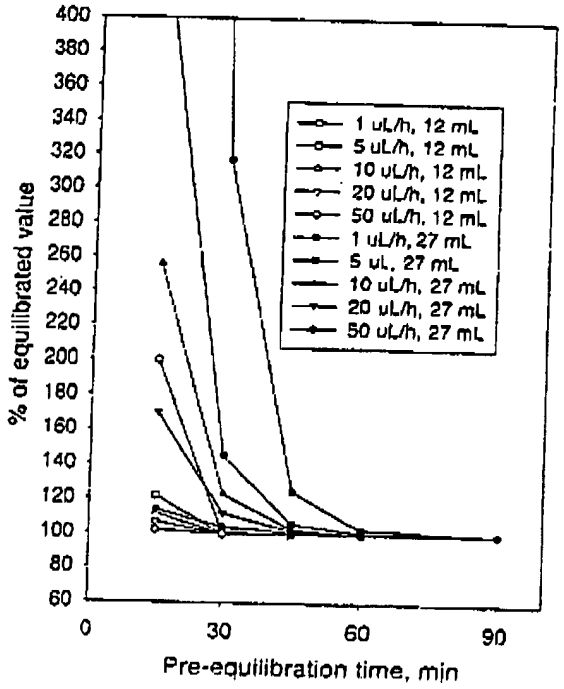

Figure 5. The relationship between the "pre-equilibration" time and the % of the equilibrated value (ratio of CO_2 absorption rate/equilibrated CO_2 absorption rate multiplied by 100) for a sample in a microrespirometer using 0.002M alkaline solution.

The minimum time required for a sample in the respirometer to reach an equilibrium was deduced from a computer simulation based on the relationship between the CO_2 absorption rate and the CO_2 concentration of the respirometer and the CO_2 evolution rate of the sample. That is, the concentration of CO_2 in the headspace of a respirometer after being shaken for a small increment of time Δt is,

$$C_{i+\Delta t} = C_i + \frac{(A_{Ci} - E)\Delta t}{V_{headspace}} \qquad (3)$$

where C_i and $C_{i+\Delta t}$ are the CO_2 concentrations of the respirometer at time i and time $i + \Delta t$, respectively. A_{Ci} is the CO_2 absorption rate of the respirometer at time i and a function of the CO_2 concentration C_i. E is the CO_2 evolution rate of the sample and $V_{headspace}$ is the volume of the headspace. The mathematical relationship of A_{Ci} and C_i was generated by a nonlinear regression curve fitting program (TableCurve, Jandel Scientific, San Rafael, CA) using the data of Figure 4. The regression enabled the calculation of A_{Ci} based on C_i. The values of A_{Ci}, C_i, and $C_{i+\Delta t}$ for each small time increment (0.5 min) of Δt were calculated and tabulated using a spreadsheet software (Excel, Microsoft, Redmond, WA) based on equation 3. An equilibrium is attained in the simulation when the CO_2 concentration in the respirometer approaches a constant, i.e., $(A_{Ci} - E)$ approaches 0 and $C_{i+\Delta t}$ approaches C_i. The minimum time required to attain an equilibrium is the sum of all small time increments, Δt, during which CO_2 concentration approaches a constant. The ratio of the CO_2 absorption rate to evolution rate (i.e., A_{Ci}/E) expressed as a percentage of the CO_2 evolution rate during the time course of reaching an equilibrium is presented in Figure 5. Two headspace volumes of the respirometer, i.e., 12 mL (5 mL remaining headspace in the sample vial plus 7 mL in the reaction chamber) and 27 mL (20 mL remaining headspace in the sample vial plus 7 mL in the reaction chamber) were simulated in Figure 5.

The results indicated that the smaller the headspace, the quicker an equilibrium is reached, and that the greater the CO_2